PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/28, A61K 39/00, 39/39 // C12N 15/31

A1

(11) International Publication Number: WO 98/45324

(43) International Publication Date: 15 October 1998 (15.10.98)

(21) International Application Number: PCT/US98/06725

(22) International Filing Date: 3 April 1998 (03.04.98)

(30) Priority Data:
60/043,410 4 April 1997 (04.04.97) US

(71) Applicant *(for all designated States except US)*: THE UAB RESEARCH FOUNDATION [US/US]; Suite 1120G, 701 South 20th Street, Birmingham, AL 35294-0111 (US).

(71)(72) Applicants and Inventors: MCGHEE, Jerry, R. [US/US]; 1038 Oak Tree Road, Birmingham, AL 35244 (US). YAMAMOTO, Shingo [JP/US]; Suite 1120G, 701 South 20th Street, Birmingham, AL 35294-0111 (US). TAKEDA, Yoshifumi [JP/US]; Suite 1120G, 701 South 20th Street, Birmingham, AL 35294-0111 (US). KIYONO, Hiroshi [JP/US]; 2937 Panorama Trail, Vestavia Hills, AL 35216 (US).

(74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

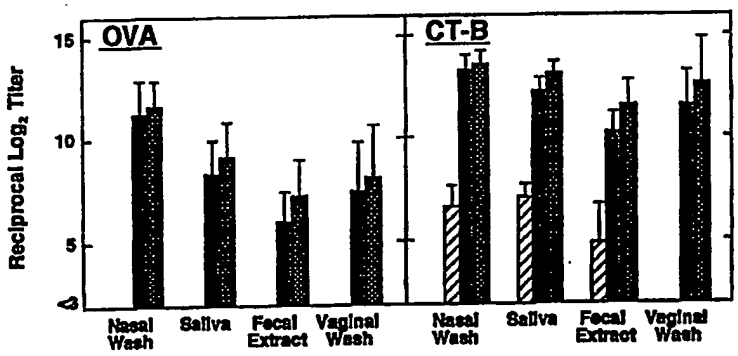
(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

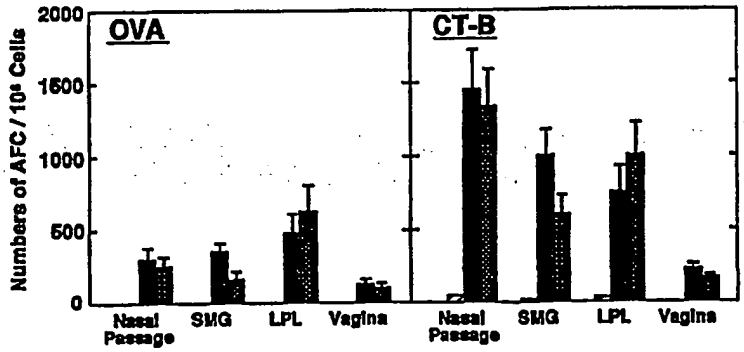
Published
With international search report.

(54) Title: USE OF CHOLERA TOXIN MUTANTS AS MUCOSAL ADJUVANTS

(57) Abstract

The invention provides a nontoxic mutant of cholera toxin that is efficacious as an adjuvant. Also provided are immunogenic compositions comprising the nontoxic mutant of cholera toxin and methods of using the immunogenic compositions.





FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

USE OF CHOLERA TOXIN MUTANTS AS MUCOSAL ADJUVANTS

Background of the Invention

The mucosal immune system can be divided into inductive sites, where vaccines/adjuvants are taken up for initial sensitization of B and T cells, and into effector sites where actual B cell and antibody responses actually occur. Oral or intranasal immunization leads to vaccine uptake into gut-associated or nasal-associated lymphoid tissues (GALT or NALT) which is followed by migration of vaccine-specific B and T cells into multiple mucosal effector sites such as the lamina propria regions of the gastrointestinal (GI), upper respiratory and genitourinary tracts as well as glandular tissues. In these effector regions polymeric IgA is produced and transported into the external secretion with specificity for the antigen initially encountered in GALT or NALT. This circular pathway, termed the Common Mucosal Immune System is where vaccine plus adjuvant are used to induce responses in a particular mucosal inductive site. These responses in turn result in antibody responses in multiple external secretions. The development and commercialization of mucosal vaccines has been hampered by the lack of an effective, nontoxic, mucosal adjuvant.

Both cholera toxin (CT) produced by *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin (LT) induce significant antibody (Ab) responses and also function as potent mucosal adjuvants for co-administered, unrelated antigens (Ags), especially when given orally (C. O. Elson et al., J. Immunol., 132, 2736-2741 (1984); C. O. Elson et al., J. Immunol., 133, 2982-2897 (1984); N. Lycke et al., Immunology, 59, 301-308 (1986); J. D. Clements et al., Vaccine, 6, 269-277 (1988)). It is known that serum IgG and IgA and mucosal IgA Ab responses are induced in mice given protein antigens orally with CT as adjuvant. It is also known that CT elicits adjuvant responses by inducing Ag-specific $CD4^+$ T cells secreting the cytokines IL-4, IL-5, IL-6 and IL-10, which characterize the so-called Th2-type (J. Xu-Amano et al., J. Exp. Med., 178, 1309-1320 (1993); M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995); J. L. VanCott et al., J. Immunol., 156, 1504-1514 (1996)). The induction of Ag-specific $CD4^+$ Th2

cells correlated directly with serum IgG1 and IgG2b subclass, IgE and mucosal IgA responses in mice orally immunized with Ag and CT as adjuvant (M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995)). A recent report (H. F. Staats et al., J. Immunol., 157, 462-472 (1996)) indicates that CT enhances serum IgG and mucosal IgA responses to a peptide antigen of HIV-1 which was poorly immunogenic when intranasally administered alone.

Both CT and LT, however, have been shown to cause severe diarrhea in humans; it has been estimated, for example, that as little as 5 micrograms of native CT given orally to an adult human would be toxic, and that a 25 microgram dose would elicit a 20 liter diarrhea. Thus, the native toxins are not suitable for use as an adjuvant in humans.

CT and LT are multi-subunit macromolecules composed of two structurally, functionally and immunologically separate A and B subunits (Figure 1). The B subunit of each toxin consists of five identical 11.6 kD peptides, but differ from each other in that the B subunit of CT (CT-B) only binds to GM1 ganglioside, while the B subunit of LT (LT-B) binds GM1 as well as asialo GM1 and GM2. Following binding of the B subunit to epithelial cell GM1 or GM2, the A subunit reaches the cytosol and following activation, binds to NAD and catalyzes ADP-ribosylation of Gsα. This GTP-binding protein activates adenylate cyclase, resulting in elevation of intracellular cyclic AMP (cAMP) levels, which in epithelial cells causes secretion of water and chloride ions into the small intestine yielding a characteristic watery diarrhea.

Since the A subunit is responsible for toxicity, it has been suggested that CT-B be used as an adjuvant. Although CT-B alone initially appeared to have adjuvant properties (S. Tamura et al., Vaccine, 6, 409-413 (1988); A. Lee et al., Infect. Immun., 62, 3594-3597 (1994)), CT-B prepared from holotoxin is now known to contain a small but sufficiently immunogenic amount of the toxic subunit CT-A (S. Spiegel, J. Cell. Biochem., 42, 143-152 (1990)).

In vitro studies in a variety of cells, e.g., B cells (N. Lycke et al., J. Immunol., 142, 3781-3787 (1989)), T cell lines (H. J. Lee et al., J. Immunol., 151, 6135-6142 (1993); E. Munoz et al., J. Exp. Med., 172, 95-103 (1990)), macrophages (A. Bromander et al., J. Immunol., 146, 2908-2914 (1991)), and

epithelial cells (D. W. McGee et al., Infect. Immun., 61, 4637-4644 (1993)), were carried out to assess the potential mechanism whereby CT and LT enhance the immune response. In most of these studies, it was concluded that adjuvanticity of CT resulted from the ADP-ribosyltransferase activity, i.e., induction of increased intracellular cAMP formation. In Bromander et al., J. Immunol., 146, 2908-2914 (1991), CT as well as forskolin was shown to inhibit T cell receptor-mediated IL-2 production and proliferation in cloned Th1 cells, but not to inhibit IL-4 production and proliferation in a clone of Th2 cells. This indicates that Th1 and Th2 cells differ in their sensitivity to increases in intracellular cAMP.

Attempts to dissociate diarrhoeagenicity of these molecules from adjuvanticity have to date been unsuccessful. For example, a mutant LT toxin E112K, which involved a single amino acid substitution in the ADP-ribosyltransferase active center (T. Tsuji et al., J. Biol. Chem., 265, 22520-22525 (1990); T. K. Sixma et al., Nature, 355, 561-564 (1992)) was nontoxic but also lacked adjuvanticity (N. Lycke et al., Eur. J. Immunol., 22, 2277-2281 (1992)). This led to the conclusion that ADP-ribosyltransferase activity was essential for adjuvanticity of both LT and CT (N. Lycke et al., Eur. J. Immunol., 22, 2277-2281 (1992)).

Two single amino acid substitution mutants of LT, R7K, (G. Douce et al., Proc. Nat'l. Acad. Sci. U.S.A., 92, 1644-1648 (1995) and R192G (B. L. Dickinson et al., Infect Immun., 63, 1617-1623 (1995)) were recently shown to be nontoxic and still retain adjuvant properties when co-administered with protein by intranasal or oral routes, respectively. However, these amino acid substitutions are located outside the ADP-ribosyltransferase cleft, and the mutants retained low ADP-ribosyltransferase activity. This potentially could cause diarrhea in humans, were the mutants to be administered orally. Another LT mutant designated S63K (M. Pizza et al., Mol. Microbiol., 14, 51-60 (1994)) was shown to be without toxicity; however, this mutant exhibited poor mucosal adjuvant properties when administered intranasally (A. Di Tommaso et al., Infect. Immun., 64, 974-979 (1996)).

Mucosal vaccines are often safer and more effective than vaccines administered subcutaneously. However, at present there are no commercially available mucosal adjuvants for use in humans. What is needed therefore is an adjuvant for use with single or multiple vaccines given by mucosal routes.

Summary of the Invention

The present invention provides a nontoxic mutant of cholera toxin that is effective as an adjuvant, preferably a mucosal adjuvant, for immunogenic compositions such as vaccines. Specifically, the present invention provides a mutant cholera toxin selected from the group consisting of S61F, E112K, a nontoxic subunit of S61F, a nontoxic subunit of E112K, a nontoxic derivative of S61F and a nontoxic derivative of E112K, wherein the nontoxic subunit or derivative is effective as an adjuvant when co-administered with an immunogenic amount of an antigen.

A "subunit" of a selected CT mutant means a continuous sequence of amino acids that is present within the full polypeptide sequence of the selected CT mutant. A "derivative" of a selected CT mutant is defined as a chemically or enzymatically altered variant of the full CT sequence or of subunit of the full sequence, such as, for example, an oxidized, reduced, amidated, esterified, or conjugated variant of S61F or E112K. The CT mutants of the present invention include variants of S61F and E112K that contain various amino acid additions, deletions, or substitutions as long as the resultant molecule is nontoxic (i.e., lacks ADP-ribosyltransferase activity) and retains substantial adjuvanticity, as described herein. Preferably, the nontoxic subunit or derivative of S61F or E112K of the invention is an effective mucosal or parenteral adjuvant, more preferably an effective mucosal adjuvant.

The cholera toxin mutants of the invention are nontoxic in that they lack ADP-ribosyltransferase activity and as a result are not diarrhoeagenic in mammals. S61F and E112K are each single-site mutants of cholera toxin wherein the single amino acid substitutions are present in the ADP-ribosyltransferase active center of the cholera toxin A subunit. Like the native cholera toxin, however, the mutant cholera toxin of the invention serves as an

effective adjuvant when co-administered with another antigen. An adjuvant is a compound that enhances the immune response mounted by the recipient against a co-administered antigen. The present cholera toxin mutants are particularly suited for use as mucosal adjuvants, and the present invention thus provides a method for use of the mutant cholera toxin as an adjuvant, preferably a mucosal adjuvant.

The invention also provides a vaccine comprising (i) the mutant cholera toxin of the invention (ii) an immunogenic amount of an antigen, and (iii) a pharmaceutically acceptable carrier. Administration of the vaccine can take any convenient form, such as parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous) or mucosal administration. Preferably, the vaccine is formulated for mucosal administration, more preferably for either oral or intranasal administration. The vaccine of the invention can be administered to any bird or mammal, and is preferably administered to a domesticated animal or a human.

Brief Description of the Figures

Figure 1 shows the amino acid sequence nCT strain 569B (A) A subunit and (B) B subunit. Amino acid differences for porcine LT (LTp) and human LT (LTh) are shown below the homologous CT sequences (B. D. Spangler, Microbiol. Rev., 56, 622-647 (1992)).

Figure 2 shows serum OVA- and CT-B-specific Ab responses following subcutaneous immunization with OVA combined with mCTs or nCT as adjuvants. Groups of C57BL/6 mice were immunized subcutaneously with 100 micrograms of OVA alone (□) or together with 10 micrograms of rCT-B (▨), 1 microgram of nCT (▩), or 10 micrograms of mCTs, S61F (▩) or E112K (■), on days 0 and 14.

Figure 3 shows OVA- and CT-B-specific $CD4^+$ T cell proliferative responses following subcutaneous immunization with OVA combined with mCTs or nCT as adjuvants. Groups of C57BL/6 mice were immunized subcutaneously with 100 micrograms of OVA alone (□) or together with

10 micrograms of rCT-B (▨), 1 microgram of nCT (▩), or 10 micrograms of mCTs, S61F (▩) or E112K (■), on days 0 and 14.

Figure 4 shows cytokine production from OVA-specific splenic $CD4^+$ T cells.

Figure 5 shows serum OVA-and CT-B-specific IgA, IgM and IgG (A) and IgG subclass (B) responses on day 21 following intranasal immunization with OVA combined with mCT S61F or nCT as adjuvants. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■), on days 0, 7 and 14.

Figure 6 shows numbers of OVA- and CT-B-specific IgG (A) and IgA (B) AFC in CLN, lung tissues and spleen following intranasal immunization with OVA combined with mCT S61F or nCT as mucosal adjuvants. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■), on days 0, 7 and 14.

Figure 7 shows OVA- and CT-B-specific IgA Ab responses in mucosal secretions following intranasal immunization with OVA combined with mCT S61F or nCT as adjuvants. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■), on days 0, 7 and 14.

Figure 8 shows OVA- and CT-B-specific $CD4^+$ T cell proliferative responses isolated from lung (A) and spleen (B) following intranasal immunization with OVA combined with mCT S61F or nCT as adjuvants. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■), on days 0, 7 and 14.

Figure 9 shows cytokine production from OVA-specific $CD4^+$ T cells isolated from lung tissue.

Detailed Description

The mutant cholera toxins of the present invention retain the high adjuvanticity of native cholera toxin but lack ADP-ribosyltransferase activity and are therefore expected to lack the diarrhoeagenicity associated with the native peptide. The mutant cholera toxins of the invention are thus suitable for use in any vaccine formulation, preferably those designed to elicit immunity via mucosal delivery, and are particularly useful as adjuvants in vaccines given either intranasally or by the oral route for induction of immunity via the Common Mucosal Immune System. Examples of human vaccines containing immunogenic antigens are found in the Mayo Clinic Family Health Book, D. Larson, M.D. ed., William Morrow & Co. Inc., pp. 338-340, 867 (1990) and R. Atlas, Microbiology Fundamentals and Applications, Macmillan Publishing Company, p. 527 (1984). Methods of preparing vaccines are well-known in the art and disclosed, for example, in U.S. Pat. No. 5,419,907 (May 30, 1995). Examples of pharmaceutically acceptable carriers and suitable dosages are listed therein at col. 5. Immunization as a means of disease prevention is described, for example, in R. Atlas, Microbiology Fundamentals and Applications, Macmillan Publishing Company, pp. 525-530 (1984).

Mutant CT S61F (mCT S61F) was made by replacing serine with phenylalanine at position 61 of the cholera toxin A subunit. Mutant CT E112K (mCT E112K) was made by replacing glutamic acid with lysine at position 112 of the A subunit. Both amino acid substitutions caused the A subunit to lose ADP-ribosyltransferase activity and diarrhoeagenicity. Both mutants supported Ag-specific responses which were comparable to native CT (nCT) when given parenterally. mCT S61F is an effective mucosal adjuvant when administered intranasally and induces mucosal and systemic Ab responses which are mediated by $CD4^+$ Th2-type cells. mCT E112K is also an effective adjuvant when administrated by either the intranasal or oral routes. Nontoxicity and adjuvanticity of other S61F and E112K CT variants of the invention can be conveniently established using the assays described herein below.

Cholera toxin mutants S61F and E112K are devoid of ADP-ribosyltransferase activity, are unable to induce increases in intracellular cAMP,

and fail to elicit fluid accumulation in mouse ligated ileal loops. Advantageously, these mCTs retain the ability of native CT to boost Ab responses to the co-administered antigenic protein ovalbumin (OVA), as well as enhance Ab responses to the CT molecule itself, e.g., to the CT-B subunit. These adjuvant effects of mCTs are presumably due to the A subunit portion of the molecule, since co-administration of rCT-B at much higher concentrations with OVA does not enhance anti-OVA Ab responses.

Native CT (nCT) is known to elicit adjuvanticity by induction of Ag-specific $CD4^+$ Th cells secreting Th2-type cytokines, which in turn provide B cell help for serum IgG1, IgG2b, IgA and IgE and mucosal S-IgA Ab responses. S61F and E112K also both induce significant OVA-specific $CD4^+$ T cell proliferative responses, with subsequent production of cytokines IL-4, IL-5, IL-6 and IL-10 (Th2-type) at levels comparable to the Th2-type response observed when nCT is used as adjuvant. The mutant CTs (mCTs) of the present invention thus retain adjuvanticity despite a lack of ADP-ribosyltransferase activity. These observations suggest that both the mCTs of the present invention and nCT enhance the immunogenicity of a co-administered, unrelated protein via the same mechanism, and that, contrary to the previous teachings in the field (N. Lycke et al., Eur. J. Immunol., 22, 2277-2281 (1992)), this mechanism is independent of the ADP-ribosyltransferase activity exhibited by nCT.

Furthermore, the IgG subclasses of the Abs induced by mCTs comprise largely IgG1 and IgG2b, and OVA-specific $CD4^+$ T cells from mice given mCT were of the Th2 type, a pattern essentially identical to that obtained with nCT. Thus, in marked contrast to the previous observations, the present findings indicate that adjuvanticity of CT can be dissociated from ADP-ribosyltransferase activity and enterotoxicity, and the $CD4^+$ Th2-type T cell responses induced by CT are due to a pathway separate from the adenyl cyclase system.

It is noteworthy that the present mCT E112K exhibits significant adjuvant activity, while a related LT mutant E112K does not (N. Lycke et al., Eur. J. Immunol., 22, 2277-2281 (1992)). CT and LT share a significant degree of homology (approximately 80% amino acid sequence identity) and some antibodies induced to CT-B cross react with LT-B and vice versa. However,

although CT and LT are both potent adjuvants, the molecules differ in terms of the nature of $CD4^+$ Th cell subsets induced and the profile, isotype and subclass of Abs induced. For example, CT elicits adjuvanticity by promoting Ag-specific $CD4^+$ Th2-type responses associated with high levels of IL-4 and IL-5 production with provision of help for IgG1 subclass, IgE and S-IgA responses (J. Xu-Amano et al., J. Exp. Med., 178, 1309-1320 (1993); M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995)), whereas LT promotes both Th1- and Th2-type responses with high levels of IFN-γ and IL-5 production and subsequent IgG1, IgG2a, IgG2b subclass and S-IgA Ab responses (I. Takahashi et al., J. Infect. Dis., 173, 627-635 (1996)). Furthermore, oral administration of CT as adjuvant failed to enhance Ag-specific S-IgA responses in IL-4 gene disrupted ($IL\text{-}4^{-/-}$) mice (M. Vajdy et al., J. Exp. Med., 181, 41-53 (1996)), whereas LT was able to induce Ag-specific mucosal S-IgA as well as serum IgG responses in both $IL\text{-}4^{-/-}$ and $IL\text{-}4^{+/+}$ mice. These differences cannot be ascribed to ADP-ribosyltransferase activity which both molecules share.

Advantageously, mCT elicits significant protein-specific IgG, IgA and IgM Ab responses in serum and elicits IgA Abs in mucosal secretions after intranasal administration, and these responses are comparable to those induced when nCT is used as mucosal adjuvant. Significant enhancement is seen with different protein components, including poorly immunogenic OVA as well as TT and influenza virus. In contrast, rCT-B fails to enhance anti-protein Ab responses in serum or in the external secretions, indicating that the A subunit of CT is necessary for adjuvant activity.

Intranasal immunization, like other mucosal routes, offers several advantages when compared to parenteral immunization. For example, lower doses of immunizing proteins are required to induce effective Ab responses when compared with other routes, and this can decrease the cost for vaccination. In order to elicit intestinal mucosal IgA responses comparable to those induced by oral immunization with CT, typically only 5-10% of the quantity of vaccine is required when given intranasally. This intranasal does also effectively induces serum IgG Ab responses when compared with parenteral immunization which required more doses of OVA and mCT or nCT as adjuvants. In addition, the

doses used in intranasal immunization induce lower total and Ag-specific IgE levels in serum than typical protocols used for oral administration (M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995)). This implies that intranasal immunization may have less risk for anaphylactic reactions. In this regard, mCT has an additional advantage over nCT, since serum CT-B-specific IgE levels elicited by mCT are significantly lower. Also, both total and OVA-specific IgE responses are less than those induced by nCT, although both molecules induce elevated Ag-specific Ab responses in serum. Further, intranasal immunization effectively induces not only systemic IgG but also mucosal IgA responses in mucosal effector tissues. Thus, intranasal vaccination using mCT could be useful in humans to prevent systemic, gastrointestinal or respiratory diseases as well as sexually transmitted diseases including HIV infection.

The mechanisms by which CT acts as a mucosal adjuvant are only partially understood. As noted above, native CT elicits adjuvanticity by induction of antigen-specific $CD4^+$ T cells secreting IL-4, IL-5, IL-6 and IL-10, i.e., Th2-type cells, which in turn effectuate for serum IgG1, IgA and IgE and mucosal IgA Ab responses. The present mCT molecules of the invention induce significant OVA-specific $CD4^+$ T cell proliferative responses, resulting in high levels of Th2-type cytokine production, which levels are comparable to those with nCT as adjuvant. On the other hand, OVA alone or OVA plus rCT-B do not induce this characteristic profile. These findings support the concept that both mCT and nCT boost Ab responses to the co-administered protein OVA through help provided by $CD4^+$ Th2-type cells. Furthermore, although rCT-B does induce detectable CT-B-specific IgA Ab responses in mucosal secretions including nasal washes, saliva and fecal extracts, it fails to elicit anti-CT-B Abs in vaginal washes. The Th2-type $CD4^+$ T cell responses elicited by mCT and nCT thus differ from those elicited by rCT-B, in that the former induce detectable responses in distant mucosal sites, such as sites within the reproductive tract system, as well as proximal mucosal sites such as the salivary gland and respiratory system sites.

The *in vitro* effects of CT on T cells has remained controversial. One study showed that cAMP activates the IL-5 promoter in EL-4, a thymoma T cell

line (H. J. Lee et al., J. Immunol., 151, 6135-6142 (1993)). On the other hand, CT has been shown to inhibit mitogen- and anti-CD3-stimulated T cell proliferative responses (D. L. Anderson et al., J. Immunol., 143, 3647-3652 (1989); (J. B. Imboden et al., Proc. Nat'l Acad. Sci. USA, 83, 5673-5677 (1986)). In another study, CT as well as forskolin were shown to inhibit T cell receptor-mediated IL-2 production and proliferation in cloned Th1 cells but not IL-4 production and proliferation in a clone of Th2 cells, indicating that Th1 and Th2 cells differ in their sensitivity to an increase in cAMP (E. Munoz et al., J. Exp. Med., 172, 95-103 (1990)). Surprisingly, however, mCT elicited serum IgG1 and IgG2b subclass and mucosal IgA Ab responses despite the lack of ADP-ribosyltransferase activity and resultant lack of cAMP induction. Further, OVA-specific $CD4^+$ T cells from mice given mCT as adjuvant yielded a clear pattern of Th2-type responses which were identical to those induced when nCT was used as adjuvant. These findings indicate that the $CD4^+$ Th2-type T cell responses induced by CT are elicited via a pathway separate from the adenyl cyclase system. CT may up-regulate of a yet to be characterized pathway which induces $CD4^+$ T cells into a Th2-type subset, and this mechanism has not been elucidated by previous *in vitro* studies. Such adjuvant properties of CT appear to be associated with the A subunit, since rCT-B do not enhance anti-OVA Ab responses in the studies disclosed herein.

The present invention is described herein with reference to various specific embodiments, examples and techniques, however it should be understood that many variations and modifications may be made while remaining within the scope of the invention.

EXAMPLES

Example 1: Construction and Purification of CT Mutants

A 3.1 kb *Eco*RI/*Pst*I DNA fragment including the CT gene from *V. cholerae* O1 strain GP14 was cloned into phage M13mp19. Single strand DNA (ssDNA) was prepared from a culture supernatant of *E. coli* CJ236 transfected with M13mp19 including the CT gene and was subjected to a site-directed mutagenesis system using Mutan K (Takara Biomedicals, Kyoto, Japan) as described in T. A. Kunkel et al., Methods Enzymol., 154, 367-382 (1987). The sequences of oligonucleotides used for the serine to phenylalanine substitution at position 61 (S61F) and for the glutamate to lysine mutation at position 112 (E112K) were 5'-GGATATGTTTTTACCTCAATT-3' (SEQ ID NO:7) and 5'-GATGAACAAAAAGTTTCTGCT-3' (SEQ ID NO:8) , respectively. The amino acid mutation sites (i.e., 61 and 112) are both in the CT-A subunit, located in the proposed ADP-ribosyltransferase active center of CT, and substitution of these amino acids in LT have been shown to completely inactivate ADP-ribosyltransferase activity and enterotoxicity (S. Harford et al., Eur. J. Biochem., 183, 311-316 (1989); T. Tsuji et al., J. Biol. Chem., 265-22520-22525 (1990)). After the DNA sequences were confirmed, pUC119 harboring the mutated CT genes at the *Eco*RI/*Pst*I site were transformed into *E. coli* DH5α. *E. coli* strains containing the plasmids for the mutant CT genes were grown in LB medium (10 g NaCl, 10 g tryptone and 5 g yeast extract/L) with 100 micrograms/ml of ampicillin, and CT mutants were purified using a D-galactose immobilized column (Pierce, Rockford, IL) from a cell suspension prepared by sonication. A plasmid containing the rCT-B gene (M. T. Dertzbaugh et al., Gene, 82, 335-342 (1989)) was kindly provided by Dr. Charles O. Elson at the University of Alabama at Birmingham and CT-B was also purified by use of a D-galactose immobilized column.

Example 2: Biologic, Enzymatic and Toxicity Assays of mCTs

Chinese hamster ovary cell morphology. The ability of CT mutants (mCTs) and native CT (nCT) to induce toxic effects in cultured Chinese hamster ovary (CHO) cells was assessed using the method of Guerrant et al. (R. L.

Guerrant, et al., Infect. Immun., 10, 320-327 (1974)). Briefly $\log_{10}$ dilutions of each toxin were added to CHO cell cultures (2×10^5 cells/0.5 ml of F10 medium containing 1% FCS) and cultured at 37° C in 5% CO_2 for 24 hours. Toxicity was defined as spindle formation in > 20% of cell cultures. For cAMP assessment, 1×10^6 CHO cells in F10 medium containing 1% FCS were cultured with 1 nanogram/ml of mCTs or nCT for 24 hours as described above. The cellular protein precipitated with 5% trichloroacetic acid was dissolved in 0.2 N NaOH and the protein amount was determined (Bio-Rad Laboratories, Hercules, CA). The supernatants were assessed for cAMP with an enzyme immunoassay (EIA) system (Amersham International, Buckinghamshire, UK), and the levels of cAMP were expressed as picomoles of cAMP/milligram of protein.

ADP-ribosyltransferase activity of mCTs. The CT-A-catalyzed transfer of ADP-ribose from NAD to agmatine was done precisely in accordance with the method of Noda et al. (M. Noda et al., Biochemistry, 28, 7936-7940 (1989)). Briefly, each assay tube contained 10 micrograms of mCTs or nCT in a total volume of 300 microliters and a 50 microliter aliquot of the assay mixture was assessed for radioactive ADP-ribosylated agmatine by liquid scintillation counting.

Assessment of toxicity using mouse ileal loops. The enterotoxicity of mCTs and nCT was examined using a mouse ileal loop test (K. Fujita et al., J. Infect. Dis., 125, 647-655 (1972)). Groups of mice were anesthetized, and 100 microliters of PBS containing different doses of each toxin were injected into a 2 cm ileal loop which was isolated by suture. The mice were sacrificed 18 hours after the injection and the ratio of fluid to length was determined and defined as positive when the ratio was more than 40 microliters/cm.

Results. As expected, as little as 1.0 picogram/ml of nCT induced spindle cell formation in CHO cell cultures, a response previously shown to be dependent upon adenyl cyclase-mediated increases in cAMP. However, neither of the mCTs affected the appearance of CHO cells even at levels of 1.0 microgram/ml. These results were confirmed by direct measurement of intracellular cAMP levels in CHO cells, which were sharply increased in nCT-treated, but not in mCT-treated cultures (Table 1). Quantitative analysis of

ADP-ribosyltransferase activity was assessed and again increased enzymatic activity was associated with nCT but not with mCTs (Table 1). The toxicity of mCTs and nCT was also assessed in a mouse ileal loop assay, where as little as 100 nanogram of nCT induced significant fluid accumulation in ligated loops, while 1,000-fold higher levels (100 micrograms) of mCTs were nontoxic (Table 1).

Table 1. *Comparison of biologic, enzymatic and toxic activity of mCTs and nCT*

Adjuvant assessed	CHO assay (pg/ml)*	cAMP induction (pmol/mg)‡	ADP-ribosyltransferase activity (cmp)§	Ileal loop test (ng)[11]
nCT	1	739 ± 127	4669 ± 256	100
S61F	$>10^6$	8.3 ± 1.8	93 ± 12	$>10^5$
E112K	$>10^6$	6.2 ± 2.2	98 ± 15	$>10^5$
PBS	–	9.7 ± 2.2	98 ± 6	–

* CHO cells were cultured in tissue culture chamber with $\log_{10}$ dilutions of each toxin for 24 hours and the toxic effects were defined as spindle formation in > 20% of cultured cells.

‡ CHO cells were cultured with 1 nanogram/ml of each toxin for 24 hours and cAMP assessed by an EIA system. The protein in 5% trichloroacetic acid precipitates was determined and concentrations of cAMP were expressed as the mean picomoles of cAMP/mg of protein ± SEM of 3 samples. The results are representative of three separate experiments.

§ The radioactivity of ADP-ribosylated agmatine induced by mCTs or nCT in a 50 microliter aliquot of the assay mixture was expressed as the mean cpm ± SEM of 6 samples. The results are representative of three separate experiments.

[11] The enterotoxicity of mCTs and nCT was examined using an ileal loop test, where mice were anesthetized, and 100 microliters of PBS containing different levels of each toxin were injected into a 2 cm ileal loop. Loops were examined 18 hours later and the ratio of fluid to length was defined as positive when the ratio was more than 40 microliters/cm.

Example 3: Immunological Response to Subcutaneous Administration of mCTs

Mice and their immunization. In order to assess the immunologic properties of mCTs, groups of mice were immunized with OVA combined with each mCT or with nCT as a control. Specifically, C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) at 5-6 weeks of age and were used at 8 to 12 weeks of age. Mice were immunized subcutaneously with 100 micrograms of OVA (Sigma Chemical Co., St. Louis, MO) alone or together with 10 micrograms of mCTs (S61F or E112K), with 1 microgram of nCT (List Biological Laboratories, Campbell, CA), or with 10 micrograms of rCT-B on days 0 and day 14.

Detection of Ag-specific Abs by ELISA and antibody-forming cells (AFCs) by enzyme-linked immunospot (ELISPOT) assay. Ab titers in serum were determined by ELISA and splenic AFCs by ELISPOT assay as described previously (J. Xu-Amano et al., J. Exp. Med., 178, 1309-1320 (1993); M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995). Endpoint titers determined by ELISA were expressed as the reciprocal $\log_2$ of the last dilution giving an optical density OD_{450} of $\geq$ 0.1 units above unimmunized controls. In the ELISPOT assay, the AFCs were determined by direct counting of spots.

Adjuvant properties of mCTs. Figure 2 shows OVA- and CT-B-specific Ab responses following subcutaneous immunization with OVA combined with mCTs or nCT as adjuvants. Serum IgM, IgG and IgA responses (A) and IgG subclass (B) responses were assessed by endpoint ELISA. Splenic Ag-specific AFCs (C) were determined by ELISPOT assay. Groups of C57BL/6 mice were immunized subcutaneously with 100 micrograms of OVA alone (□) or together with 10 micrograms of rCT-B (▨), 1 microgram of nCT (▩), or 10 micrograms of mCTs, S61F (▩) or E112K (■), on days 0 and 14. All assays were performed on samples from mice taken 1 week after the last immunization. Bars represent the mean Ab titer and mean number of AFCs ± SEM in each group of 10 mice and the data are representative of three separate experiments.

Immunization with OVA alone did not result in significant anti-OVA Ab responses, and admixture of OVA with rCT-B also failed to support anti-OVA

Ab responses; however, both mCTs and nCT enhanced serum anti-OVA Abs and these responses were mainly of the IgG isotype (Fig. 2A). Further, anti-OVA Abs were largely restricted to IgG1 with less IgG2b subclass response (Fig. 2B). Significant numbers of splenic OVA-specific IgG AFCs were noted in mice given OVA combined with mCTs or with nCT, whereas low numbers of AFCs were observed in mice given OVA alone or OVA with rCT-B (Fig. 2C). Thus, both mCTs induced an Ab pattern remarkably similar to the serum Ab responses which resulted from use of nCT as adjuvant. Mice immunized with OVA and mCTs or nCT as adjuvant also showed significant anti-CT-B-specific IgG responses. Although IgG anti-CT-B responses were also seen in mice given rCT-B, the titers were approximately 100-fold lower than seen when either mCTs or nCT were given (Fig. 2A). In addition, mCTs and nCT induced high levels of anti-CT-B specific IgG1 and IgG2b Abs (Fig. 2B). Large numbers of CT-B-specific IgG AFCs were present in splenic cells from mice immunized with mCTs or with nCT, while much lower numbers of AFCs were seen in mice immunized with rCT-B (Fig. 2C).

IgE analysis. Past studies have shown that CT induces marked increases in both total and Ag-specific IgE Abs following mucosal immunization (M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995)). Two sensitive assays were used to detect increased total serum IgE as well as Ag-specific IgE Abs in mice given OVA combined with mCTs or with nCT as adjuvant. Total IgE levels were determined by ELISA as described in the preceding paragraph. Ag-specific IgE was detected by a modified IgE capture method (M. Sakaguchi et al., J. Immunol. Methods, 190, 189-197 (1989)). Briefly, 96 well microplates (Dynatech Microlite, Chantilly, VA) were coated with 1 microgram/ml of rat anti-mouse IgE mAb (PharMingen, San Diego, CA) in 50 mM carbonate-bicarbonate buffer (pH 9.5). After blocking with 3% BSA-PBS, serial dilutions of serum were added. Following incubation and washing, 2.5 microgram/ml of biotinylated-OVA or 1.5 microgram/ml of CT-B were added in 3% BSA-PBS-Tween 20. The plates were then washed with 2 mM EGTA-PBS-Tween 20 and incubated with 10 nanogram/ml streptaequorin (SeaLite Sciences, Inc., Bogard, GA) in 2 mM EGTA-PBS-Tween 20. Light development was carried out in a

Dynatech ML-3000 luminometer by injection of Ca^{2+} buffer (50 mM Tris, 20 mM calcium acetate, pH 7.5) (R. J. Jackson et al., J. Immunol. Methods, 190, 189-197 (1996)). Endpoint titers were determined as the dilution of each sample showing a 2-fold higher level of luminometric units above background.

Maximum IgE responses peaked by 3 weeks and although differences in total IgE levels were not significant in mice given mCTs or nCT (Table 2), anti-OVA IgE titers were lower in mice given mCT S61F ($p < 0.05$), whereas CT-B-specific IgE Abs were depressed in mice given mCTs S61F or E112K when compared with nCT ($p < 0.01$) (Table 2). In mice given OVA alone or OVA plus rCT-B, neither total nor Ag-specific IgE responses were noted.

Table 2. *IgE responses induced by mCTs and nCT*

Treatment group*	Total IgE (ng/ml)‡	Ag specific-IgE (reciprocal $\log_2$ titer)‡ OVA	CT-B
OVA alone	128 ± 32	3.5 ± 0.9	<3
OVA + rCT-B	134 ± 46	3.6 ± 1.1	<3
OVA + nCT	1408 ± 402	9.4 ± 0.7	5.5 ± 0.8
OVA + S61F	1267 ± 416	7.3 ± 0.9§	3.1 ± 0.3[11]
OVA + E112K	1086 ± 313	7.8 ± 0.7	<3[11]

* Mice were immunized subcutaneously with 100 micrograms of OVA alone or together with 10 micrograms of rCT-B, 1 microgram of nCT, or 10 micrograms of mCTs, S61F or E112K, on days 0 and 14. Each group contained 10 mice. The results are representative of three separate experiments

‡ IgE responses on day 21 were determined by ELISA (total) and luminometric assay (Ag-specific).

§ Significantly lower ($p < 0.05$ when compared with nCT).

[11] Significantly lower ($p < 0.01$ when compared with nCT).

OVA- and CT-B-specific splenic CD4+ T cell responses. Single spleen cell suspensions in complete RPMI 1640 medium were fractionated on a nylon

wool column for 1 hour at 37°C to remove adherent cells. The $CD4^+$ T cell subset (>98% purity) was then obtained by positive sorting using a magnetic bead activated cell separation system (MACS, Miltenyi Biotec Inc., Sunnyvale, CA) using biotinylated anti-CD4 mAb (GK1.5) and streptavidin coated microbeads (Miltenyi Biotec Inc.). Purified splenic $CD4^+$ T cells were cultured at a density of 2×10^6 cells/ml with 1 mg/ml of OVA or with 1×10^7 CT-B-coated beads/ml, T cell-depleted, irradiated (3000 rads) splenic feeder cells (2.5×10^6 cells/ml), and IL-2 (10 Units/ml) (PharMingen) in complete medium (Xu-Amano et al., J. Exp. Med., 178, 1309-1320 (1993); M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995); J. L. VanCott et al., J. Immunol., 156, 1504-1514 (1996)). As positive controls, $CD4^+$ T cells from nonimmunized mice were stimulated with a solid-phase anti-mouse CD3 mAB (145-2C11). To measure cell proliferation, 0.5 microcuries of [Methyl-^{3}H]-thymidine (Dupont NEN Products, Boston, MA) was added to individual culture wells 18 hours before termination, the cells were harvested and the radioactivity was assessed by liquid scintillation counting after 96 hours of culture. To determine cytokine production by Ag-specific $CD4^+$ T cells, the cells were harvested after 48 hours of culture for quantitative reverse transcriptase (RT)-PCR analysis of cytokine-specific mRNA and the supernatants were collected after 96 hours for evaluation of cytokines by ELISA. For IL-2, supernatants from 48 hour cultures were used since this represented the interval for maximal production of this cytokine.

Cytokines in culture supernatants were determined by a modified ELISA (M. Marinaro et al., J. Immunol., 155, 4621-4629 (1995)). Nunc-ImmunoMaxiSorp™ plates were coated with 2.5 microgram/ml of anti-mouse IFN-γ, IL-2, IL-4, IL-5, IL-6 or IL-10 mAB (PharMingen). For secondary Abs and detection enzyme, 0.2 microgram/ml of biotinylated rat anti-mouse cytokine mAb (PharMingen) and 1:4000 diluted horseradish peroxidase-labeled anti-biotin (Vector Laboratories, Burlingame, CA) were used, respectively.

Quantitative cytokine-specific RT-PCR using rRNA internal standards was conducted as described in M. Yamamoto et al. and T. Hiroi et al. (M. Yamamoto et al., Am. J. Pathol., 148, 331-339 (1996); T. Hiroi et al., Eur. J. Immunol., 25, 2743-2751 (1995). Cytokine-specific rRNA for IFN-γ, Il-2, Il-4,

IL-5, IL-6 or IL-10 were used as internal standards. For quantitation, aliquots of total RNA were added with a series of diluted rRNA internal standards and standard RT-PCR was performed. Analysis of PCR products were conducted by capillary electrophoresis with a laser induced fluorescence detection system (CE-LIF) (LIF-P/ACE, Beckman Instruments, Fullerton, CA).

OVA- and CT-B-specific $CD4^+$ T cell proliferative responses are shown in Figure 3. Groups of C57BL/6 mice were immunized subcutaneously with 100 micrograms of OVA alone (□) or together with 10 micrograms of rCT-B (▨), 1 microgram of nCT (□), or 10 micrograms of mCTs, S61F (▩) or E112K (■), on days 0 and 14. Purified splenic $CD4^+$ T cells were cultured at a density of 2×10^6 cells/ml in the presence of 1 mg/ml of OVA or 1×10^7 CT-B-coated beads/ml, T cell depleted, irradiated splenic feeder cells (2.5×10^6 cells/ml) and IL-2 (10 U/ml) in complete medium. Bars represent the mean stimulation index ± SEM in each group. Each group contained 10 mice and are representative of three separate experiments. Figure 4 shows cytokine production from OVA-specific splenic $CD4^+$ T cells. Molecules of cytokine-specific mRNA were determined by quantitative RT-PCR using rRNA internal standards. Cytokine production was determined by ELISA. The scale of each figure corresponds to mRNA molecules and protein levels produced by nonimmunized $CD4^+$ T cells stimulated with anti-CD3 mAb. Bars represent the mean cytokine-profile ± SEM in each group. ND indicates that the molecules were not detected. Each group contained 5 mice and are representative of three separate experiments.

Both mCTs were found to induce OVA-specific $CD4^+$ T cell proliferative responses, which were comparable to those seen in mice given nCT as adjuvant. Moreover, splenic OVA-specific $CD4^+$ T cells from mice given OVA together with mCTs produced high levels of Th2-type cytokines (IL-4, IL-5, IL-6 and IL-10), which were comparable to those seen when nCT was used as adjuvant (Fig. 4). On the other hand, $CD4^+$ T cells from mice given OVA alone or OVA plus rCT-B did not produce detectable levels of cytokines other than IFN-γ when stimulated with OVA. Abundant Th2-type cytokine-specific mRNA was present in OVA-specific $CD4^+$ T cell cultures taken from mice given OVA combined with mCTs or with nCT, but was not detected in $CD4^+$ T cells from mice given

OVA alone or OVA plus rCT-B (Fig. 4). Further, IFN-γ was detected at low levels in all cultures including OVA-stimulated controls from unimmunized mice. Splenic CT-B-specific $CD4^+$ T cells from mice given mCTs or nCT also demonstrated significant proliferation (Fig. 3) and high levels of Th2-type with low but detectable levels of Th1-type cytokines (IFN-γ and IL-2). The quantitative RT-PCR results together with levels of secreted cytokines were consistent with previous studies which showed that nCT induces $CD4^+$ Th2-type responses.

The results are expressed as the mean ± SEM. Statistical significance ($p < 0.05$) was determined by Student's *t* test and by the Mann-Whitney U test of unpaired samples.

Example 4: mCT S61F as a Mucosal Adjuvant

Purification of mCT and recombinant CT-B (rCT-B). *E. coli* strains containing the plasmids for mCT S61F (Example 1) or rCT-B (M. T. Dertzbaugh et al., Gene, 82, 335-342 (1989)) genes were grown in LB medium (10 g NaCl, 10 g tryptone and 5 g yeast extract/L) with 100 microgram/ml of ampicillin. The mCT and rCT-B were purified using a D-galactose immobilized column (Pierce, Rockford, IL) from a cell suspension prepared by sonication of the bacteria according to the method of Uesaka et al. (Y. Uesaka et al., Microbial Pathogenesis, 16, 71-76 (1994)). Purity of the molecules was assessed on SDS/PAGE and no contaminating protein bands were noted following silver staining. When ADP-ribosyltransferase activity was examined in the presence of 5 micrograms of ADP-ribosylation factor (ARF) in a total volume of 300 microliters as described in Example 2, no ADP-ribosylation was observed with 4 micrograms of mCT S61F (111 ± 14.5 cpm/50 microliters of reaction mixture) or with PBS (113 ± 6.3 cpm), while the activity of 4 micrograms of nCT (List Biological Laboratories, Campbell, CA) was significantly enhanced with ARF (5101 ± 380 cpm) when compared with nCT only (1735 ± 69 cpm).

Immunization and sample collection. C57BL/6 mice were obtained from the Charles River Laboratories (Wilmington, DL) at 5-6 weeks of age and were used at 8-12 weeks of age in this study. Mice were intranasally immunized with

a 20 microliter aliquot (10 microliters per nostril) containing 100 micrograms of OVA (Sigma Chemical Co., St. Louis, MO) alone or together with 0.1, 1 or 5 microgram of mCT, 0.1 or 0.5 microgram of nCT, or 5 microgram of rCT-B on days 0, 7 and 14. Further, 25 micrograms of tetanus toxoid (TT) (kindly provided from Dr. Y. Mukai, Osaka University, Biken Foundation, Osaka, Japan) or formalin-treated influenza virus (B. Yamagata) (2 micrograms of hemagglutinin [HA]-equivalent) (kindly provided from Drs. R. B. Couch and I. N. Mbawuike at Baylor College of Medicine, Houston, TX) were immunized intranasally together with 5 micrograms of rCT-B, 0.5 micrograms of nCT or 5 micrograms of mCT S61F using the same protocol as employed for OVA. Nasal and vaginal washes were collected by gently flushing the nasal passage or vaginal canal with 20 microliters of 50 microliters of sterile PBS, respectively. Saliva was obtained following intraperitoneal injection of mice with 100 microliters of 1 mg/ml pilocarpine (Sigma). Fecal extract samples were obtained by adding weighed pellets to PBS containing 0.1% sodium azide (1 ml/100 mg fecal sample). The pellet was vortexed, centrifuged, and the supernatants were collected for assay.

Cell isolation. Cervical lymph nodes (CLN) and spleen were aseptically removed and single cell suspensions were obtained. The nasal passage, submandibular gland (SMG), lung or vaginal tissues were carefully excised, teased apart, and dissociated using collagenase type IV (Sigma) in Joklik-modified medium (Life Technologies, Inc., Gaithersburg, MD). After removal of Peyer's patches, the small intestine was stirred in PBS containing 1 mM EDTA at 37° C for 30 minutes and the lamina propria lymphocytes (LPL) were subsequently isolated using collagenase type IV. The mononuclear cells were obtained at the interface of the 40 and 75 % layers of a discontinuous percoll gradient (Pharmacia, Uppsala, Sweden) (K. Fujihash et al., J. Exp. Med., 183, 1929-1935 (1996)); T. Hiroi et al., Eur. J. Immunol., 25, 2743-2751 (1995)).

Detection of Ag-specific Ab titers by ELISA and AFC by ELISPOT assay. Ab titers in serum and mucosal secretions were determined by ELISA as described in Example 2. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving an optical density at 450 nm (OD_{450}) of $\geq$ 0.1 units above

negative controls. Enumeration of Ag-specific AFC from various tissues was performed by ELISPOT assay. The color reaction was developed with 1.6 mM 3-amino-9-ethyl carbazole in 0.1 M sodium acetate buffer (pH 5.0) containing 0.05% H_2O_2 at room temperature for 1 hour. The plates were washed with water and dried, and AFC were quantitated with the aid of a stereomicroscope.

Figure 5 shows serum OVA- and CT-B-specific IgA, IgM and IgG and IgG subclass responses on day 21 following intranasal immunization with OVA combined with mCT S61F or nCT as adjuvants were determined by endpoint ELISA. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■), on days 0, 7 and 14. Serum samples were collected 1 week after the last immunization. Bars represent the mean Ab titer ± 1 SE in each group. Each group consisted of 5 mice and the data are representative of three separate experiments. Neither OVA alone nor admixture of OVA with 5 micrograms of rCT-B elicited detectable serum anti-OVA IgM or IgA Ab responses, although low IgG Ab responses were detected (Fig. 5A). In preliminary dose response studies, admixture of 0.1 - 1 microgram of mCT S61F or 0.1 microgram of nCT induced OVA-specific IgG Abs in serum and elevated numbers of OVA-specific IgG and IgA AFC in lung tissues and spleen; however, these doses of adjuvants did not elicit optimal OVA-specific IgA Ab responses in saliva, fecal extracts or vaginal washes.

On the other hand, mice intranasally immunized with OVA plus 5 micrograms of mCT S61F or 0.5 microgram of nCT showed significantly high serum Ab titers of OVA-specific IgG, IgA, and IgM isotypes (Fig. 5A) and elevated IgG1 and IgG2b subclass responses (Fig. 5B). Assessment of levels and isotype of AFC responses also revealed significant numbers of splenic OVA-specific IgG AFC in mice given OVA with mCT or nCT as mucosal adjuvants, whereas only low numbers of AFC were observed in mice given OVA alone or OVA together with rCT-B (Fig. 6A). Significant OVA-specific IgG and IgA AFC responses were also observed in CLN and in lung tissue cell isolates of mice immunized with OVA and mCT as well as from mice given nCT as mucosal adjuvant (Fig. 6B). Only low AFC responses occurred in mice given

OVA alone or OVA together with rCT-B. Figure 6 shows numbers of OVA- and CT-B-specific IgG (A) and IgA(B) AFC in CLN, lung tissues and spleen following intranasal immunization with OVA combined with mCT S61F or nCT as mucosal adjuvants were determined by ELISPOT assay. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■) on days 0, 7 and 14. Samples were collected 1 week after the last immunization. Bars represent the mean numbers of AFC ± 1 SE and each group contained 5 mice. The data are representative of three separate experiments.

It should be emphasized that significant OVA-specific IgA Ab responses were seen in multiple mucosal secretions of mice given OVA and mCT as adjuvant. In Figure 7, OVA- and CT-B-specific IgA Ab responses in mucosal secretions were determined by ELISA (A) and numbers of IgA AFC in mucosal tissues by ELISPOT assay (B) following intranasal immunization with OVA combined with mCT S61F or nCT as mucosal adjuvants. Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 5 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■) on days 0, 7 and 14. Tissue samples and external secretions were taken 1 week after the last immunization. Bars represent the mean Ab titer or numbers of AFC ± 1 SE in each group. Each group contained 5 mice and the data are representative of three separate experiments. Anti-OVA IgA Abs were seen in nasal and vaginal washes, saliva and fecal extracts of mice immunized with OVA and mCT or nCT as adjuvants, while OVA alone or OVA plus rCT-B failed to elicit detectable IgA anti-OVA responses in any mucosal secretion (Fig. 7A). These results were consistent with AFC analyses which demonstrated significant numbers of OVA-specific IgA AFC in these mucosal effector tissues from mice given OVA and mCT or nCT, whereas AFCs were not seen in mice given OVA alone or OVA together with rCT-B (Fig. 7B).

To demonstrate that mCT S61F is an effective adjuvant for more conventional vaccines, groups of mice were also immunized intranasally with tetanus toxoid (TT) or with influenza virus mixed with mCT, nCT or rCT-B. In

these studies, the mCT as well as nCT enhanced Ab responses to TT and to influenza virus. Ag-specific IgG, IgA and IgM Ab responses in serum and Ag-specific IgA Ab responses in mucosal secretions were significantly enhanced in mice given mCT or nCT as adjuvants (Table 3). Furthermore, single cell analyses showed that significant numbers of Ag-specific IgG and IgA AFC in spleen, CLN and lung tissues and Ag-specific IgA AFC in mucosal sites of mice immunized with mCT or nCT as adjuvants. On the other hand, rCT-B failed to act as adjuvant for co-administrated TT or influenza virus (Table 3).

Table 3. *Antibody Responses in Serum and in Mucosal Secretions Following Intranasal Immunization of Mice with TT or Influenza Virus and mCT S61F or nCT as Mucosal Adjuvants*

Antigen Used*	Adjuvant*	Serum Isotypes ‡ (reciprocal $\log_2$ titer)			Mucosal IgA† (reciprocal $\log_2$ titer)			
		IgA	IgM	IgG	Nasal Wash	Saliva	Fecal Extract	Vaginal Wash
TT	None	7	9	10	3	4	<3	<3
	rCT-B	7	9	11	4	4	<3	<3
	nCT	10	14	17	8	7	7	6
	S61F	11	13	18	9	8	8	5
Influenza Virus	None	9	10	13	8	9	7	<3
	rCT-B	10	10	13	8	9	7	<3
	nCT	13	12	18	13	10	10	7
	S61F	12	13	19	14	11	10	7

*Each group comprised 5 mice and the results are representative of two separate experiments.

†Ag-specific Ab titers in serum and in mucosal secretions on day 21 were determined by endpoint ELISA.

A remarkable characteristic of CT is that in addition to adjuvant properties, it possesses strong immunogenicity, especially to the CT-B component. In this regard, mice immunized with OVA together with mCT or nCT showed high CT-B-specific IgG, IgA and IgM Ab responses (Fig. 5A), and IgG1 and IgG2b subclass anti-CT-B Ab responses in serum (Fig. 5B). Anti-CT-B Ab responses were also seen in mice given rCT-B, but were lower in magnitude when compared with mCT or nCT, even though a higher dose of rCT-B was used. Single cell analyses revealed significant numbers of CT-B-specific IgG and IgA AFC in spleen, CLN and lung tissue cell isolates from mice co-immunized with OVA plus mCT or nCT, with lower numbers of AFC in mice given rCT-B (Fig. 6). CT-B specific IgA Ab responses were also elevated in mucosal secretions, i.e., nasal and vaginal washes, saliva and fecal extracts of mice given mCT or nCT (Fig. 7).

Detection of total and Ag-specific IgE in serum. Two sensitive assays were used to detect increased total serum IgE as well as Ag-specific IgE Abs in mice given mCT or nCT as adjuvants. Total IgE levels were determined by ELISA using mouse IgE mAb (clone 27-74, PharMingen, San Diego, CA) as standard. For primary and secondary Abs, rat anti-mouse IgE mAb (clone R35-72, PharMingen) and biotinylated rat anti-mouse IgE mAb (clone R35-92, PharMingen) were employed, respectively. Ag-specific serum IgE was detected by a modified IgE-capture luminometric assay as described in Example 2. Light development was carried out in a dynatech ML-3000 luminometer by injection of Ca^{2+} buffer (50 mM Tris, 20 mM calcium acetate, pH 7.5). Endpoint titers were determined as the dilution of each sample showing a 2-fold higher level of luminometric units above background.

IgE levels peaked 2 weeks after the initial immunization. Differences in total and OVA-specific IgE levels were noted between the groups given mCT and nCT as adjuvants, but the differences were not significant. CT-B-specific IgE levels in mice given mCT were significantly lower than seen with nCT (Table 4).

Table 4. *Serum IgE Responses Induced by Intranasal Immunization with OVA and Either mCT S61F or nCT as Mucosal Adjuvants.*

		Ag specific-IgE (reciprocal $\log_2$ titer)†	
Treatment* Group	Total IgE† (ng/ml)	OVA	CT-B
OVA alone	186 ± 108	3.99 ± 1.25	<3
OVA +rCT-B	159 ± 83	3.63 ± 0.51	<3
OVA + nCT	1094 ± 237	9.14 ± 0.79	6.22 ± 0.84
OVA + S61F	768 ± 218	8.10 ± 1.05	4.24 ± 0.88 ‡

* Each group contained 5 mice and the results are representative of three separate experiments.

† Serum IgE reached maximum levels at two weeks following the initial immunization and were determined by ELISA (for total IgE) or by IgE-capture luminometric assay (for Ag-specific IgE).

‡ Significantly lower when compared with nCT ($p<0.05$).

OVA- and CT-B-specific CD4+ T cell responses. Single cell suspensions were isolated from lung tissues and spleen in complete medium (RPMI 1640 supplemented with 10 ml/L of nonessential amino acids solution, 1 mM sodium pyruvate, 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 100 Units/ml penicillin, 100 micrograms/ml streptomycin, 40 micrograms/ml gentamicin and 10% fetal calf serum (FCS). Isolated cells were fractionated on a nylon wool column for 1 hour at 37° C to remove adherent cells. The $CD4^+$ T cell subset was the obtained by positive-sorting using a magnetic bead separation system consisting of biotinylated anti-CD4 mAb (clone GK1.5) and streptavidin microbeads (MACS, Miltenyi Biotec Inc., Sunnyvale, CA) (22). Purified splenic $CD4^+$ T cells (> 98% purity) were cultured at a density of 2×10^6 cells/ml with OVA (1 mg/ml) or with CT-B-coated beads (10^7 beads/ml). The cultures also contained T cell-depleted, irradiated (3000 rads) splenic feeder cells (2.5×10^6 cells/ml) and IL-2 (10 Units/ml) (PharMingen) in complete medium. Purified $CD4^+$ cells from nonimmunized mice were stimulated with solid-phase anti-CD3 mAb (145-

2C11) or with the antigens used in the intranasal immunization as positive or negative controls, respectively.

To measure Ag-specific $CD4^+$ T cell proliferative responses, 100 microliters of culture in 96-well culture plates (Corning Glass Works, Corning, NY) were incubated at 37° C in 5% CO_2 and 10 microliters of 50 microCurie/ml [methyl-^{3}H]-thymidine was added to each well 18 hours before termination. Cells were harvested onto glass fiber filters for measurement of radioactivity by liquid scintillation counting after 96 hours of culture. To determine cytokine production by Ag-specific $CD4^+$ T cells, 1 ml of culture was incubated in 24-well cell culture plates (Coster Corp., Cambridge, MA) at 37° C in 5% CO_2 and cells were harvested after 48 hours of culture for quantitative reverse transcriptase-PCR (RT-PCR) analysis of cytokine-specific mRNA. Supernatants were collected after 96 hours for evaluation of cytokines by ELISA. For IL-2 analysis by ELISA, supernatants from 48 hour cultures were used.

Detection of cytokines by ELISA. Cytokines in culture supernatants were determined by ELISA (6, 7). Briefly, Nunc-ImmunoMaxiSorp™ plates were coated with 2.5 micrograms/ml of rat anti-mouse IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 mAb (PharMingen) as in Example 3. Serial dilutions of culture supernatants or standard cytokines (PharMingen) were added in duplicate. For secondary Ab and detection enzymes, 0.2 micrograms/ml of biotinylated rat anti-mouse cytokine mAb (PharMingen) and 1:40000 diluted horseradish peroxidase-labeled anti-biotin (Vector Laboratories, Burlingame, CA) were employed as in Example 3. The ELISA assays were capable of detecting 20 picograms/ml for IFN-γ, 0.1 Unit/ml for IL-2, 10 picograms/ml for IL-4, 2 Unit/ml for IL-5, 1 nanogram/ml for IL-6 and 0.5 nanogram/ml for IL-10.

Quantitative RT-PCR analysis of cytokine-specific mRNA. Cytokine-specific RT-PCR and quantitative analysis of RT-PCR products by capillary electrophoresis were done as described in Example 3 with minor modifications. Briefly, total RNA was isolated by the acid guanidinium thiocyanate phenol chloroform extraction procedure and subjected to standard RT-PCR. RT products with a series of diluted recombinant DNA internal standards were amplified by PCR and quantitative analysis of RT-PCR products was conducted

by capillary electrophoresis with a laser-induced fluorescence detection system (LIF-P/ACE, Beckman Instruments).

Figure 8 shows OVA- and CT-B-specific $CD4^+$ T cell proliferative responses isolated from lung (A) and spleen (B). Groups of C57BL/6 mice were immunized with 100 micrograms of OVA alone (□) or together with 10 micrograms of rCT-B (▨), 0.5 microgram of nCT (▩), or 5 micrograms of mCT, S61F (■) on days 0, 7 and 14. Purified $CD4^+$ cells were cultured at a density of 2×10^6 cells/ml in the presence of 1 mg/ml of OVA or 10^7/ml of CT-B-coated beads, T cell depleted, irradiated splenic feeder cells (2.5×10^6 cells/ml) and IL-2 (10 U/ml). Bars represent the mean stimulation index ± 1 SE and each group contained 5 mice. The data were similar and are representative of four separate experiments. Culture of $CD4^+$ T cells from lung tissues or spleen with either OVA or CT-B resulted in significant proliferative responses clearly indicating the presence of both OVA-specific and CT-B-specific $CD4^+$ Th cells in mice which had received intranasal administration of either mCT or nCT (Fig. 8).

Figure 9 shows the results of assessment of cytokine production form OVA-specific $CD4^+$ T cells isolated from lung tissue. Molecules of cytokine-specific mRNA were determined by quantitative RT-PCR using recombinant internal standards. Cytokine protein production was determined by ELISA. The scale of each figure corresponds to mRNA molecules and protein levels produced by nonimmunized $CD4^+$ T cells stimulated with anti-CD3 mAb. ND indicates not detected. Bars represent the mean cytokine profile ± 1 SE in each group. The data are representative of four separate experiments. It is apparent that OVA-specific $CD4^+$ T cells from lung tissues of mice given OVA and mCT released high levels of Th2-type cytokines (IL-4, IL-5, IL-6 and IL10) into the culture. These responses were comparable to those obtained when nCT was used as adjuvant; however, cytokine responses produced by $CD4^+$ T cells from mice given OVA alone or OVA plus rCT-B were detectable but considerably lower than observed with mCT or nCT. Cytokine-specific mRNA was examined by quantitative RT-PCR in RNA extracts from OVA-specific $CD4^+$ T cell cultures. Again, Th2-type cytokine-specific mRNA was readily detected in OVA-specific

$CD4^+$ T cells. Again, Th2-type cytokine-specific mRNA was readily detected in OVA-specific $CD4^+$ T cells from lung tissues of mice given OVA with mCT or nCT as mucosal adjuvants. Much lower levels of these cytokines were noted in cultures from mice given OVA alone or OVA plus rCT-B. On the other hand, Th1-type cytokines (IFN-γ and IL-2) were detectable at low levels in all samples including controls from nonimmunized mice. CT-B-specific $CD4^+$ T cells from lung tissues of mice given OVA plus mCT or nCT also exhibited high levels of Th2-type and low levels of Th1-type cytokines. Splenic OVA- or CT-B-specific $CD4^+$ T cells from mice given mCT or nCT also showed high levels of Th2-type cytokines by both ELISA and quantitative RT-PCR.

Results are reported as mean ± one standard error (SE). Statistical significance ($p < 0.05$) was determined by Student's t test and by the Mann-Whitney U test of unpaired samples.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The UAB Research Foundation

(ii) TITLE OF INVENTION: USE OF CHOLERA TOXIN MUTANTS AS MUCOSAL ADJUVANTS

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schwegman, Lundberg, Woessner & Kluth, P.A.
(B) STREET: 121 South Eigth Street, 1600 TCF Tower
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unknown
(B) FILING DATE: 03-APR-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/043410
(B) FILING DATE: 04-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Embretson, Janet E
(B) REGISTRATION NUMBER: 39,665
(C) REFERENCE/DOCKET NUMBER: 557.004WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-373-6959
(B) TELEFAX: 612-339-3061
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 240 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asn Asp Asp Lys Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp Glu Ile
 1               5                  10                  15
Lys Gln Ser Gly Gly Leu Met Pro Arg Gly Gln Ser Glu Tyr Phe Asp
            20                  25                  30
Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr
        35                  40                  45
Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr Ser Ile
    50                  55                  60
Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser Gly His
65                  70                  75                  80
Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe Asn
                85                  90                  95
Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Gln Glu
            100                 105                 110
Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr
        115                 120                 125
Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg Gly
    130                 135                 140
Tyr Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala Ala Asp
145                 150                 155                 160
Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Arg Glu
                165                 170                 175
Glu Pro Trp Ile His His Ala Pro Pro Gly Cys Gly Asn Ala Pro Arg
            180                 185                 190
Ser Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu Gly Val
        195                 200                 205
Lys Phe Leu Asp Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser
    210                 215                 220
Gly Tyr Gln Ser Asp Ile Asp Thr His Asn Arg Ile Lys Asp Glu Leu
225                 230                 235                 240
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 240 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asn Gly Asp Arg Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp Glu Ile
 1               5                  10                  15
Lys Arg Ser Gly Gly Leu Met Pro Arg Gly His Asn Glu Tyr Phe Asp
            20                  25                  30
Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr
        35                  40                  45
Gln Thr Gly Phe Val Arg Tyr Asp Asp Gly Tyr Val Ser Thr Ser Leu
    50                  55                  60
Ser Leu Arg Ser Ala His Leu Ala Gly Gln Ser Ile Leu Ser Gly Tyr
65                  70                  75                  80
Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe Asn
```

```
                85                  90                  95
Val Asn Asp Val Leu Gly Val Tyr Ser Pro His Pro Tyr Glu Gln Glu
            100                 105                 110
Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr
        115                 120                 125
Arg Val Asn Phe Gly Val Ile Asp Glu Arg Leu His Arg Asn Arg Glu
    130                 135                 140
Tyr Arg Asp Arg Tyr Tyr Arg Asn Leu Asn Ile Ala Pro Ala Glu Asp
145                 150                 155                 160
Gly Tyr Arg Leu Ala Gly Phe Pro Pro Glu His Gln Ala Trp Arg Glu
                165                 170                 175
Glu Pro Trp Ile His His Ala Pro Asn Gly Cys Gly Asn Ser Ser Arg
            180                 185                 190
Thr Ile Thr Gly Asp Thr Cys Asn Glu Glu Thr Gln Asn Leu Ser Thr
        195                 200                 205
Ile Tyr Leu Arg Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser
    210                 215                 220
Asp Tyr Gln Ser Glu Val Asp Ile Tyr Asn Arg Ile Arg Asp Glu Leu
225                 230                 235                 240
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln
 1               5                  10                  15
Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala
            20                  25                  30
Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asn Gly Ala Thr Phe
        35                  40                  45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala
65                  70                  75                  80
Lys Val Glu Lys Leu Cys Val Trp Asn Asn Lys Thr Pro His Ala Ile
                85                  90                  95
Ala Ala Ile Ser Met Ala Asn
            100
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu Tyr His Arg Thr Gln
 1               5                  10                  15
Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr Thr Glu Ser Met Ala
            20                  25                  30
Gly Lys Arg Glu Met Val Ile Ile Glu Ile Lys Ser Gly Glu Thr Phe
        35                  40                  45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr Tyr Leu Thr Glu Thr
65                  70                  75                  80
Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr Pro Asn Ser Ile
                85                  90                  95
Ala Ala Ile Ser Met Lys Asn
            100
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu Tyr His His Thr Gln
 1               5                  10                  15
Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr Thr Glu Ser Met Ala
            20                  25                  30
Gly Lys Arg Glu Met Val Ile Ile Thr Ile Lys Ser Gly Ala Thr Phe
        35                  40                  45
Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala
    50                  55                  60
Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr Tyr Leu Thr Glu Thr
65                  70                  75                  80
Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr Pro Asn Ser Ile
                85                  90                  95
Ala Ala Ile Ser Met Glu Asn
            100
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGATATGTTT TTACCTCAAT T 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATGAACAAA AAGTTTCTGC T 21

WHAT IS CLAIMED IS:

1. A mutant cholera toxin selected from the group consisting of S61F, E112K, a nontoxic subunit of S61F, a nontoxic subunit of E112K, a nontoxic derivative of S61F and a nontoxic derivative of E112K, wherein the nontoxic subunit or derivative is effective as an adjuvant when co-administered to a bird or mammal in combination with an immunogenic amount of antigen.

2. The mutant cholera toxin of claim 1 which is S61F or E112K.

3. The mutant cholera toxin of claim 1 which is S61F.

4. The mutant cholera toxin of claim 1 which is E112K.

5. A vaccine comprising (i) the mutant cholera toxin of claim 1 or 2, (ii) an immunogenic amount of an antigen, and (iii) a pharmaceutically acceptable carrier.

6. The vaccine of claim 5 formulated for mucosal administration.

7. The vaccine of claim 6 formulated for intranasal or oral administration.

8. The vaccine of claim 5 formulated for human administration.

9. A method comprising administering to a human a mucosal adjuvant comprising the mutant cholera toxin of claim 1 or 2.

10. A method comprising administering to a human the vaccine of claim 5 or 6.

FIG. 1A

A

```
                                             10                                      20                                      30
(CTA1)  Asn Asp Asp Lys Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp Glu Ile Lys Gln Ser Gly Gly Leu Met Pro Arg Gly Gln Ser Glu Tyr
(LTpA1)     Gly     Arg                                                     Arg                                 His Asn

                                             40                                      50                                      60
(CTA1)  Phe Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val
(LTpA1)                                                                                                 Tyr

                                             70                                      80                                      90
(CTA1)  Ser Thr Ser Ile Ser Leu Arg Ser Ala Met Leu Val Gly Gln Thr Ile Leu Ser Gly His Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr
(LTpA1)             Leu                             Ala         Ser                 Tyr

                                            100                                     110                                     120
(CTA1)  Ala Pro Asn Met Phe Asn Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Gln Glu Val Ser Ala Leu Gly Gly Ile Pro
(LTpA1)                                                 Val                     Tyr

                                            130                                     140                                     150
(CTA1)  Tyr Ser Gln Ile Tyr Gly Trp Tyr Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg Gly Tyr Arg Asp Arg Tyr Tyr
(LTpA1)                                         Asn             Ile         Arg                     Glu

                                            160                                     170                                     180
(CTA1)  Ser Asn Leu Asp Ile Ala Pro Ala Ala Asp Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Arg Glu Glu Pro Trp Ile
(LTpA1) Arg         Asn                 Glu             Arg                         Asp     Gln

                                            190                  A2                 200                                     210
(CTA1)  His His Ala Pro Pro Gly Cys Gly Asn Ala Pro Arg Ser Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu Gly Val Lys Phe
(LTpA1)                 Gln                 Ser Ser     Thr Ile Thr Gly Asp         Asn     Glu         Asn     Ser Thr Ile Tyr

                                            220                                     230                                     240
(CTA2)  Leu Asp Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser Gly Tyr Gln Ser Asp Ile Asp Thr His Asn Arg Ile Lys Asp Glu Leu
(LTpA2)     Arg                                                 Asp             Glu Val     Ile Tyr             Arg
```

FIG. 1B

B

```
                                        10                                      20                                      30
(CTB)  Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser Tyr Thr Glu Ser
(LTpB) Ala         Thr         Glu         Ser         Arg                 Tyr     Ile                 Leu
(LThB) Ala         Ser         Glu         Ser         His                 Tyr     Ile                 Leu

                                        40                                      50                                      60
(CTB)  Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe Lys Asx Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser
(LTpB) Met                         Val         Glu Ile     Ser     Glu
(LThB) Met                         Val             Ile     Ser

                                        70                                      80                                      90
(CTB)  Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn
(LTpB)                                                         Thr                 Thr     Ile Asp
(LThB)                                                         Thr                 Thr     Ile Asp

                                        100
(CTB)  Lys Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn
(LTpB)             Asn Ser                         Lys
(LThB)             Asn Ser                         Glu
```

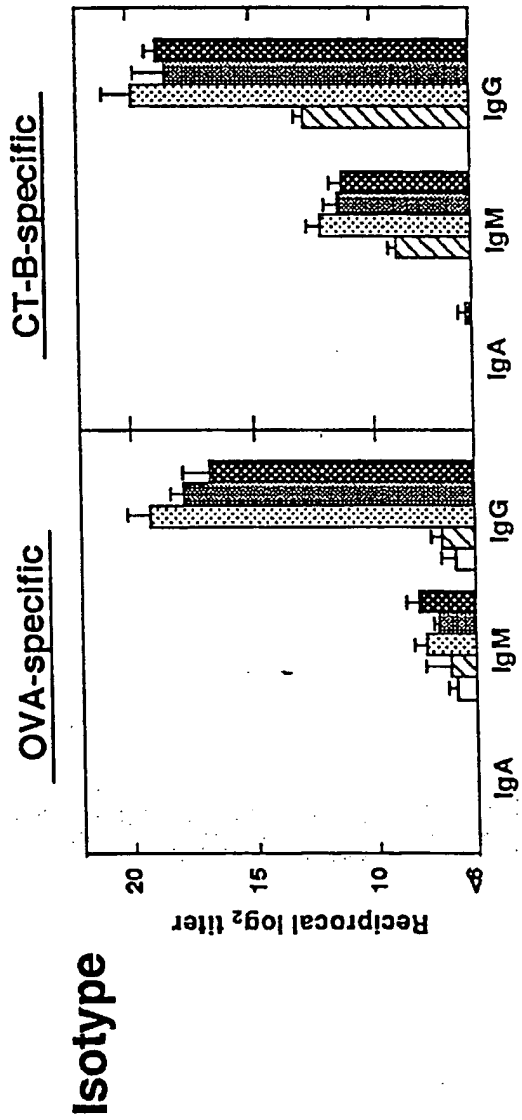

CT-B-specific
OVA-specific
IgG
IgM
IgA
IgG
IgM
IgA
20
15
10
8
Reciprocal log2 titer
Isotype

FIG. 2A

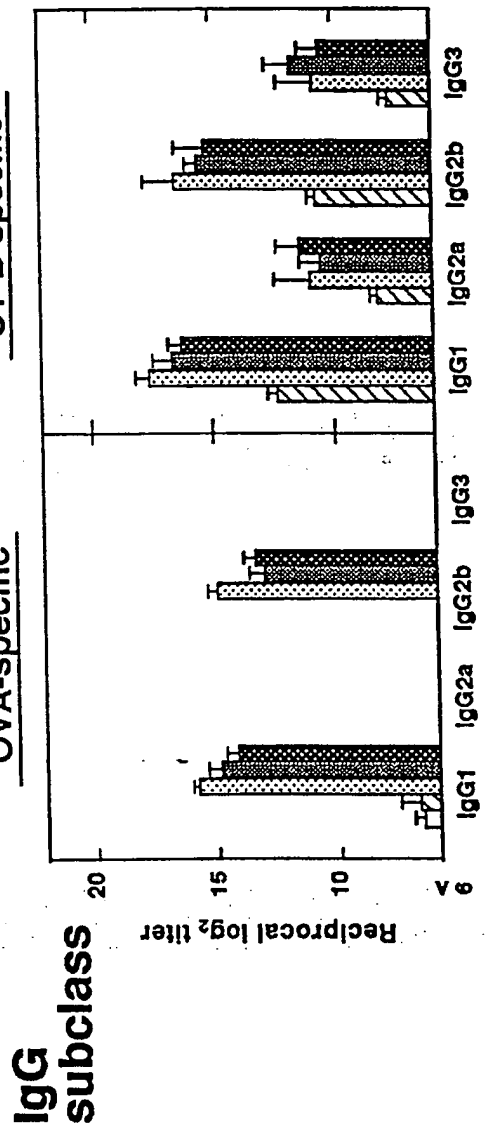

IgG subclass
OVA-specific
CT-B-specific
IgG1
IgG2a
IgG2b
IgG3
Reciprocal log2 titer
20
15
10
6

FIG. 2B

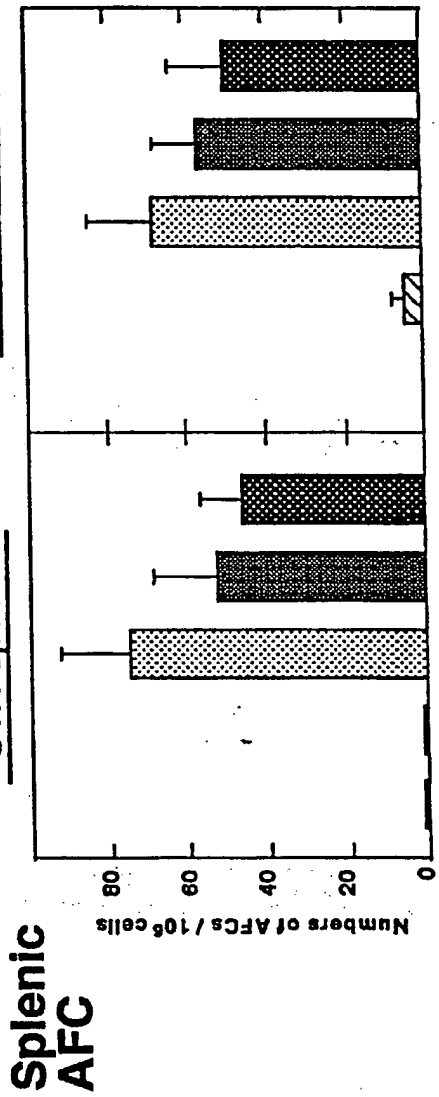

Splenic AFC
OVA-specific
CT-B-specific
Numbers of AFCs / 10^6 cells
0
20
40
60
80

FIG. 2C

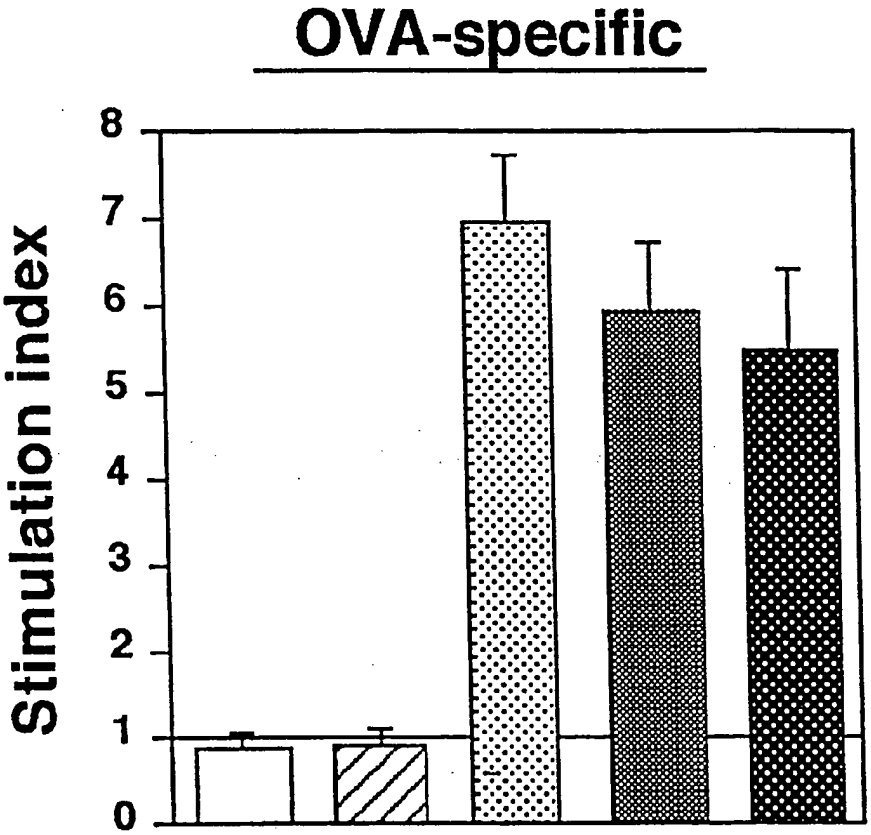


FIG. 3A

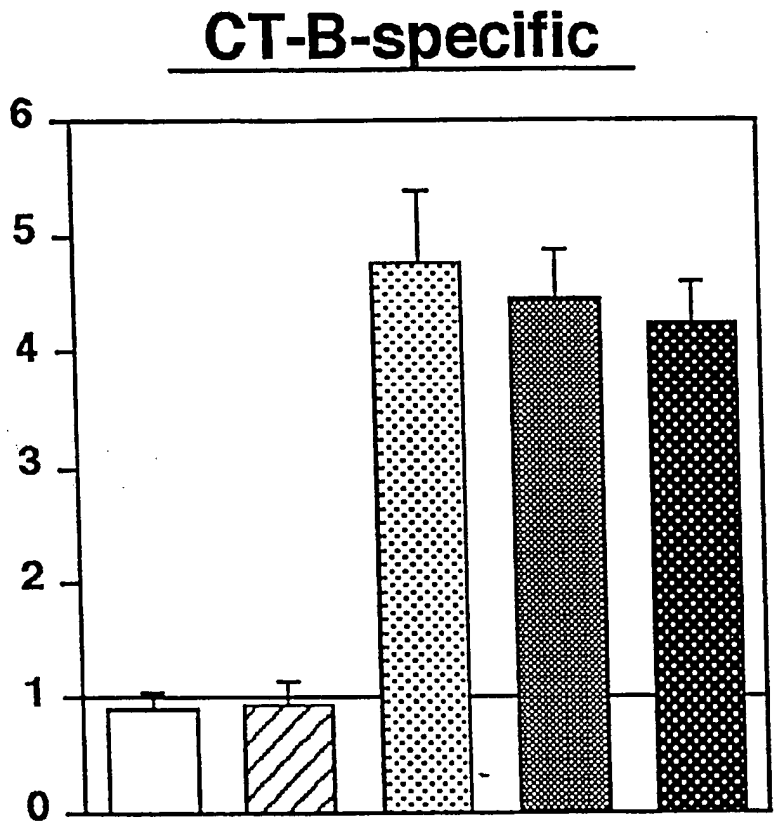


FIG. 3B

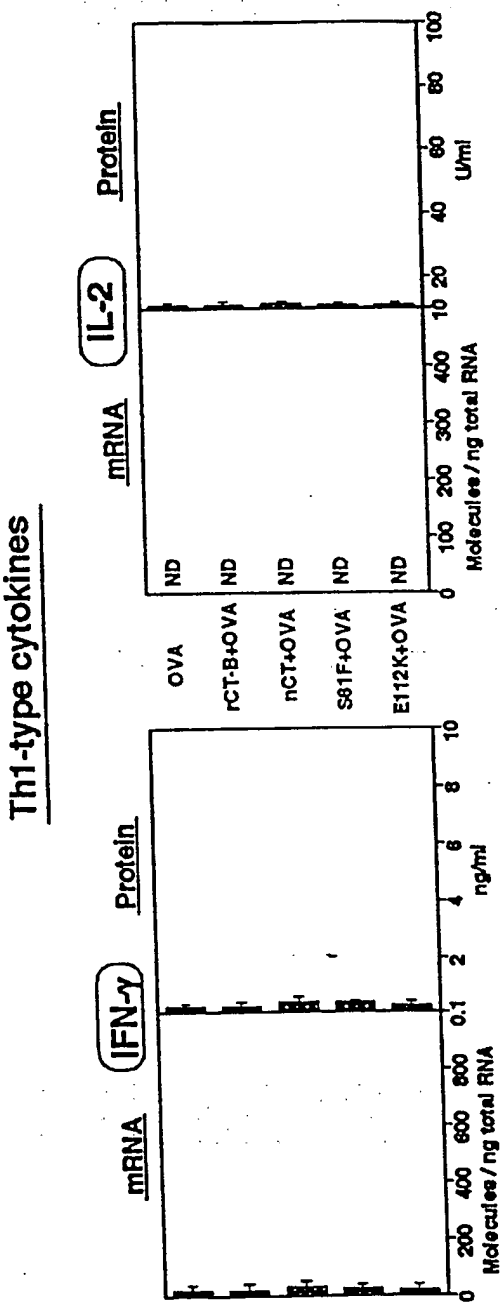

Th1-type cytokines
mRNA
IFN-γ
Protein
Molecules / ng total RNA
ng/ml
mRNA
IL-2
Protein
Molecules / ng total RNA
U/ml
OVA
rCT-B+OVA
nCT+OVA
S61F+OVA
E112K+OVA
ND

FIG. 4A

FIG. 4B

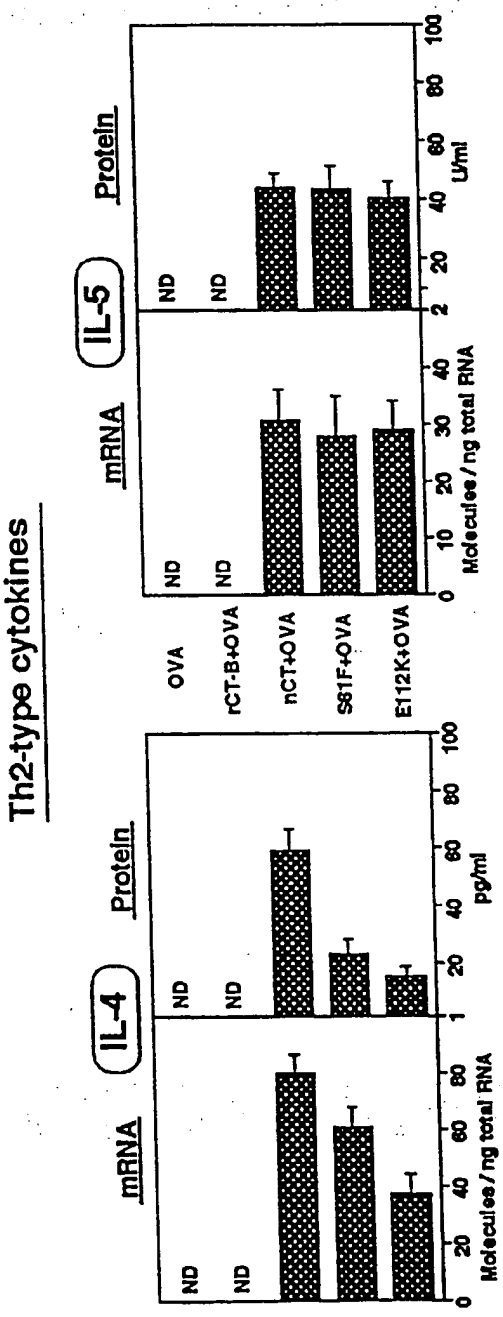

Th2-type cytokines
IL-4
mRNA
Protein
IL-5
mRNA
Protein
ND
ND
OVA
rCT-B+OVA
nCT+OVA
S61F+OVA
E112K+OVA
Molecules / ng total RNA
pg/ml
U/ml

FIG. 4C

FIG. 4D

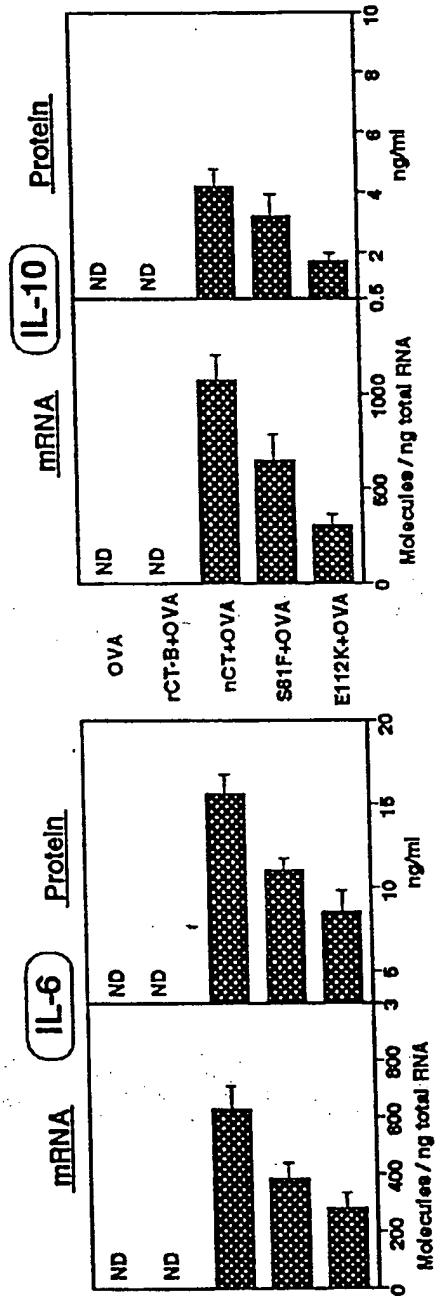

IL-10
mRNA
Protein
ND
ND
0
600
1000
Molecules / ng total RNA
0.5
2
4
6
8
10
ng/ml
OVA
rCT-B+OVA
nCT+OVA
S61F+OVA
E112K+OVA
IL-6
mRNA
Protein
ND
ND
0
200
400
600
800
Molecules / ng total RNA
3
5
10
15
20
ng/ml

FIG. 4F

FIG. 4E

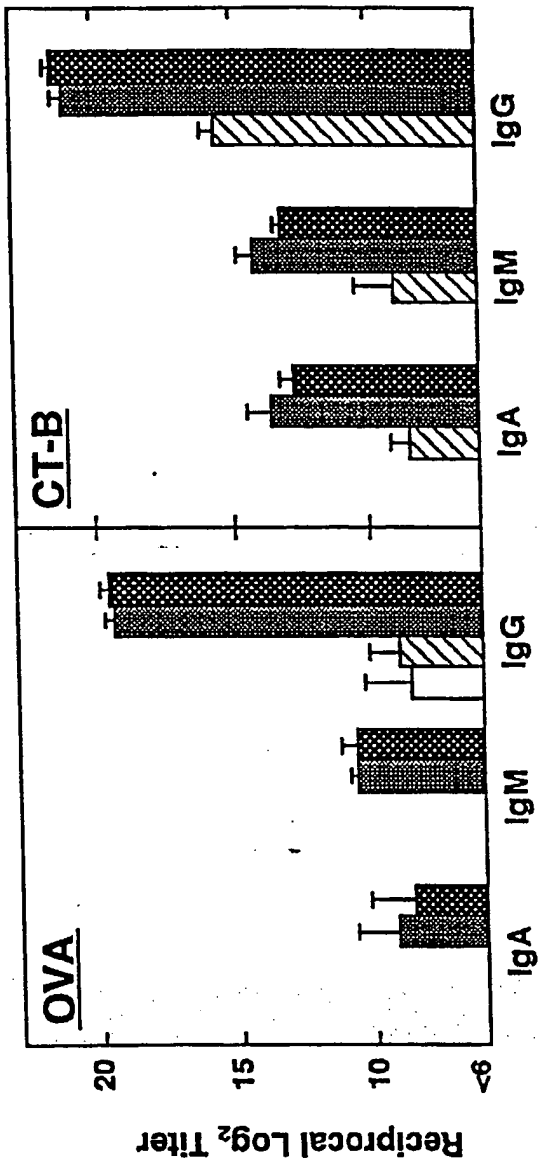

CT-B
OVA
IgG
IgM
IgA
IgG
IgM
IgA
20
15
10
<6
Reciprocal Log2 Titer

FIG. 5A

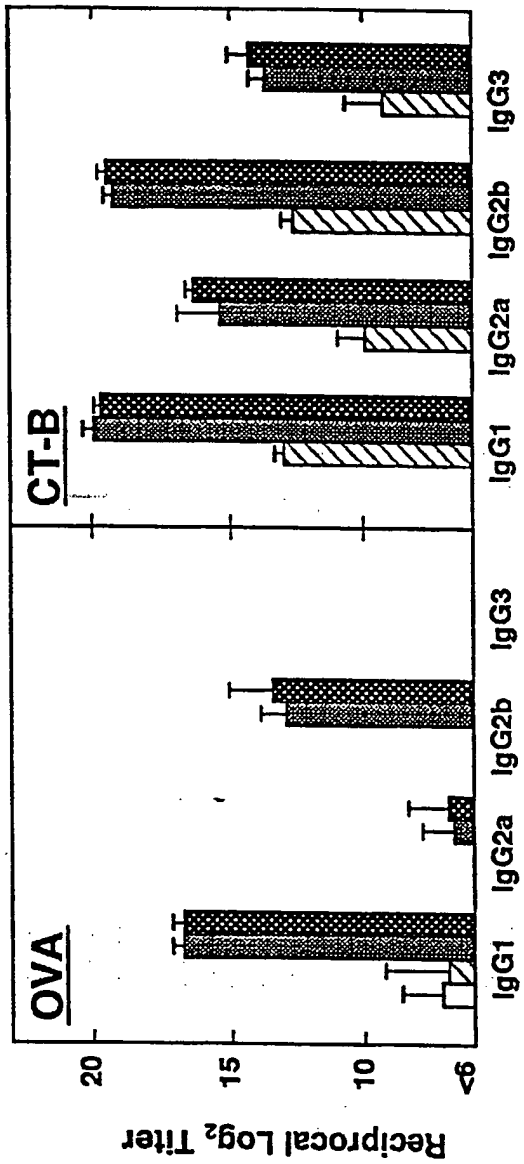

Reciprocal Log2 Titer
20
15
10
<6
OVA
CT-B
IgG1
IgG2a
IgG2b
IgG3

FIG. 5B

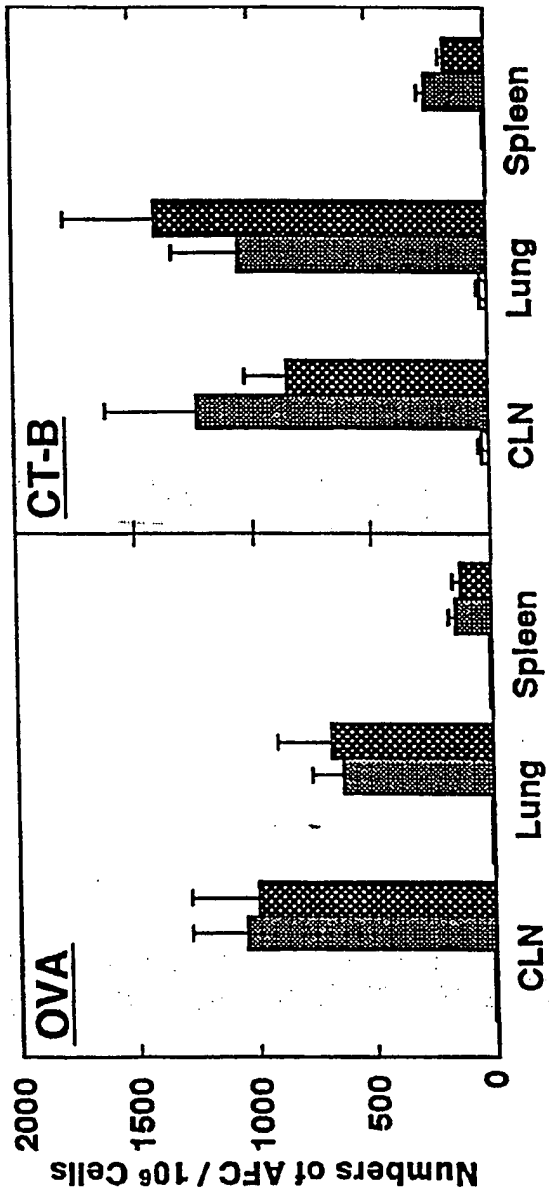

Numbers of AFC / 10⁶ Cells
2000
1500
1000
500
0
OVA
CT-B
CLN
Lung
Spleen
CLN
Lung
Spleen

FIG. 6A

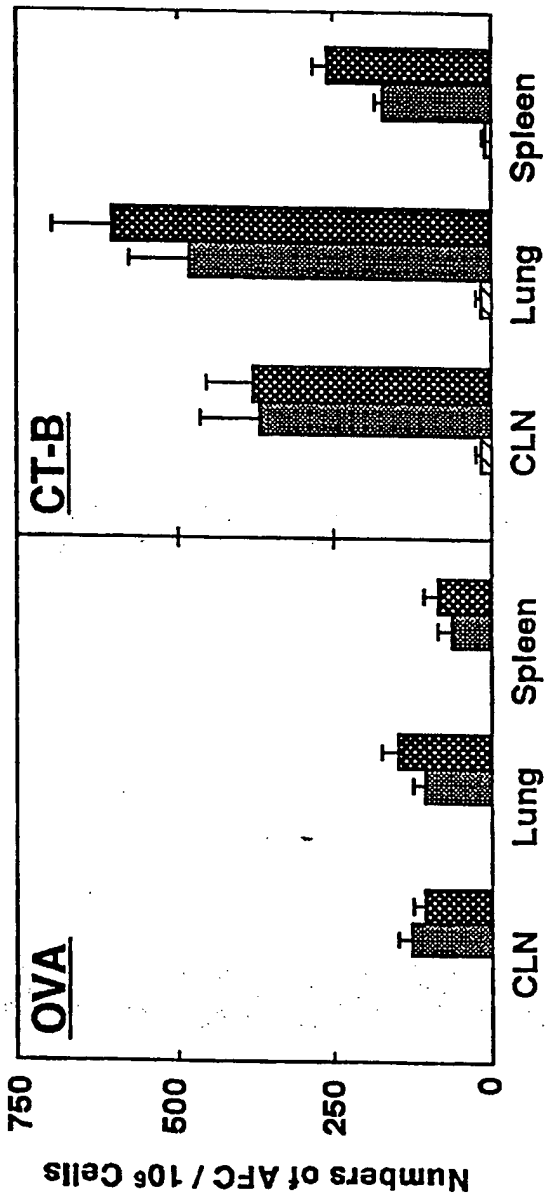

Numbers of AFC / 10⁶ Cells
750
500
250
0
OVA
CT-B
CLN
Lung
Spleen
CLN
Lung
Spleen

FIG. 6B

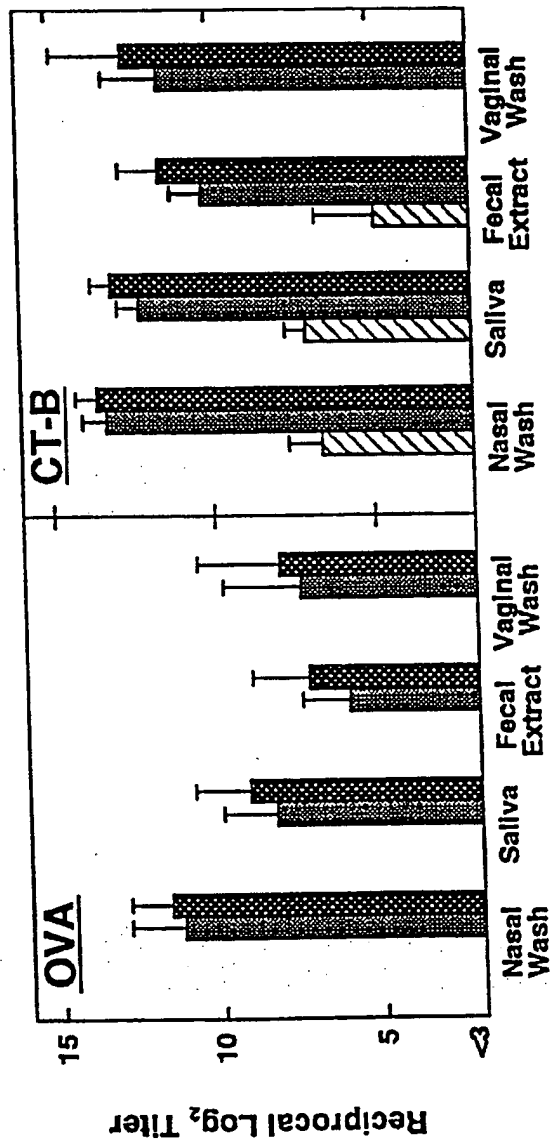

Reciprocal Log2 Titer
15
10
5
<3
OVA
CT-B
Nasal Wash
Saliva
Fecal Extract
Vaginal Wash
Nasal Wash
Saliva
Fecal Extract
Vaginal Wash

FIG. 7A

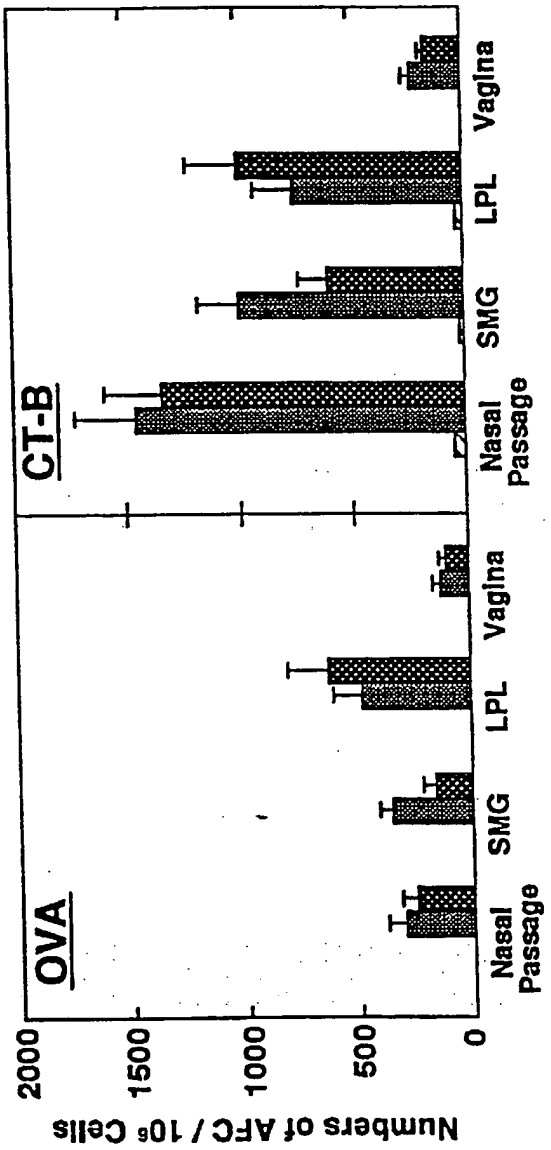

Numbers of AFC / 10⁶ Cells
2000
1500
1000
500
0
OVA
CT-B
Nasal Passage
SMG
LPL
Vagina
Nasal Passage
SMG
LPL
Vagina

FIG. 7B

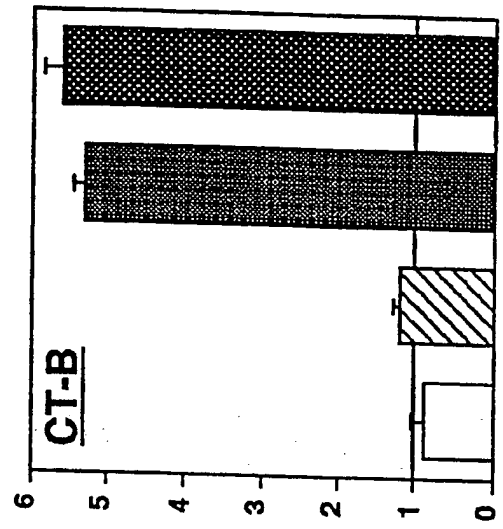


FIG. 8B

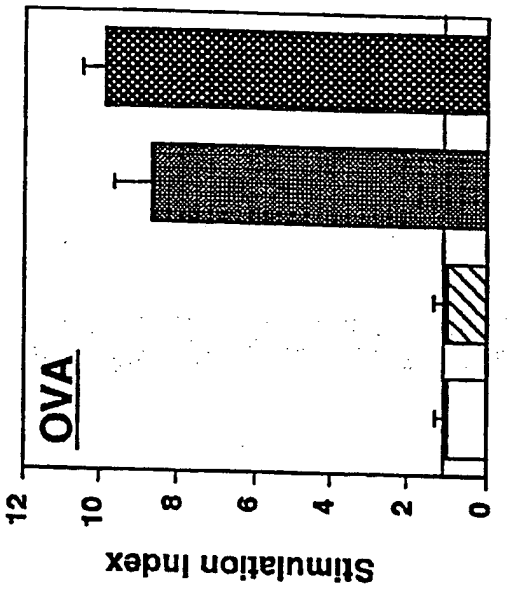


FIG. 8A

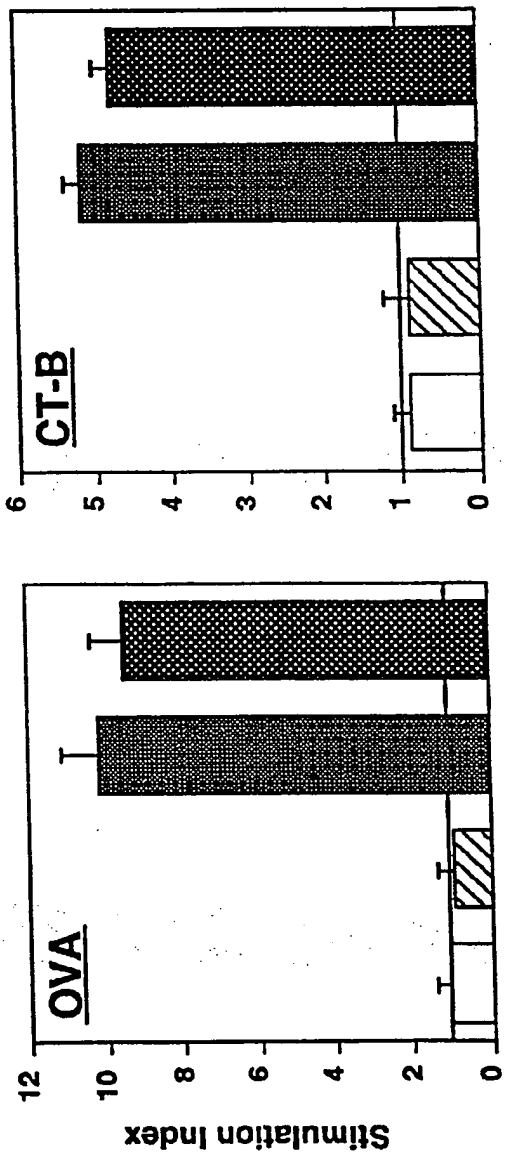


FIG. 8D

FIG. 8C

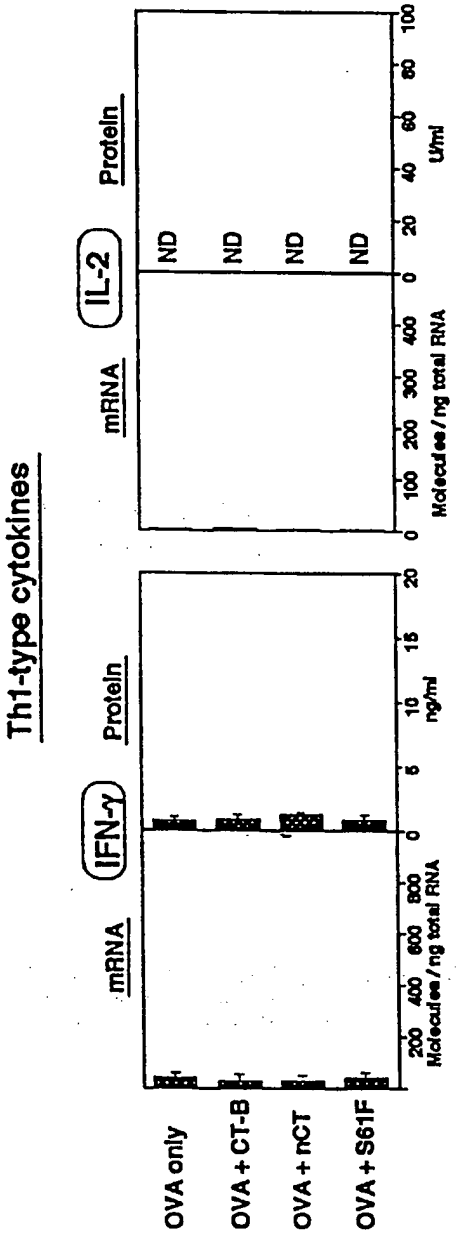

Th1-type cytokines
IFN-γ
mRNA
Protein
IL-2
mRNA
Protein
Molecules / ng total RNA
ng/ml
U/ml
ND
OVA only
OVA + CT-B
OVA + mCT
OVA + S61F

FIG. 9A

FIG. 9B

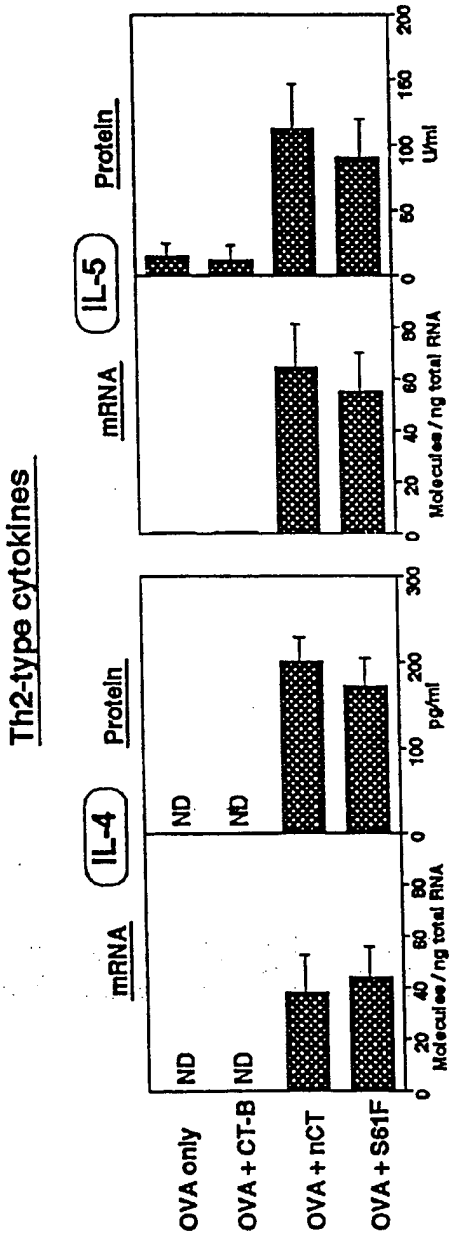

Th2-type cytokines
IL-4
IL-5
mRNA
Protein
ND
ND
Molecules / ng total RNA
pg/ml
U/ml
OVA only
OVA + CT-B
OVA + nCT
OVA + S61F

FIG. 9C

FIG. 9D

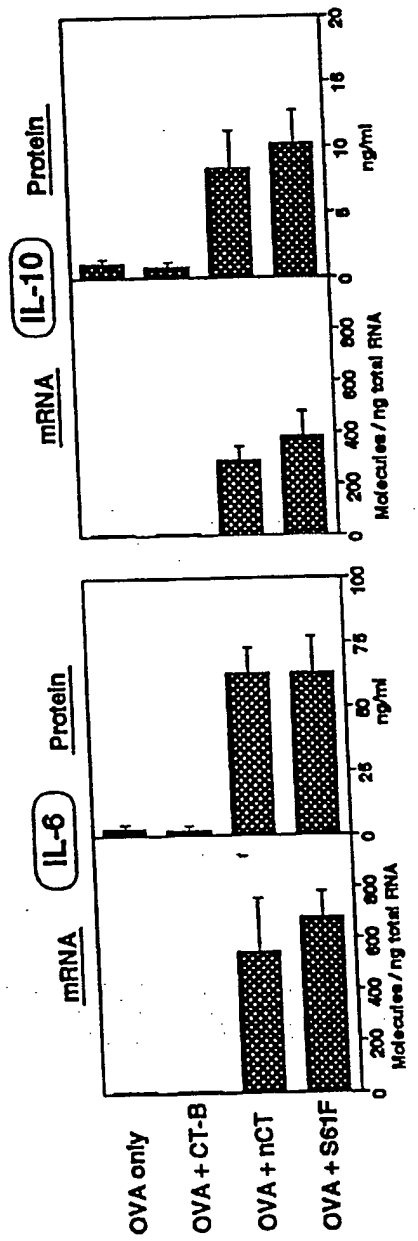

IL-10
Protein
mRNA
ng/ml
Molecules / ng total RNA
IL-6
Protein
mRNA
ng/ml
Molecules / ng total RNA
OVA only
OVA + CT-B
OVA + nCT
OVA + S61F

FIG. 9F

FIG. 9E

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/06725

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/28 A61K39/00 A61K39/39 //C12N15/31

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 02348 A (BIOCINE SPA ;PIZZA MARIAGRAZIA (IT); FONTANA MARIA RITA (IT); GIAN) 23 January 1997 see page 4, line 10 - line 24 see page 5, line 31 - page 6, line 13 see page 8, line 35 - page 9, line 2 see page 15, line 33 - page 17, line 2	1-3,5-10
Y	---	4
Y	HÄSE C.C. ET AL.: "Construction and characterization of recombinant Vibrio cholerae strains producing inactive cholera toxin analogs" INFECTION AND IMMUNITY, vol. 62, no. 8, August 1994, pages 3051-3057, XP002070088 see the whole document	4
	--- -/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1998	16.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 98/06725

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 182 109 A (TAMURA SHINICHI ET AL) 26 January 1993 see column 1, line 22 - line 31 see column 5, line 2 - line 4 see claims 1,3,4,6,7	1-3,5-10
Y	HARFORD S. ET AL. : "Inactivation of the Escherichia coli heat-labile enterotoxin by in vitro mutagenesis of the A-subunit gene" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 183, no. 2, August 1989, pages 311-316, XP002070089 cited in the application see page 315, line 43 - line 55	1-3,5-10
P,X	WO 97 29771 A (CHIRON S P A ;FONTANA MARIA RITA (IT); PIZZA MARIAGRAZIA (IT); RAP) 21 August 1997 see page 4, line 6 - page 5, line 2 see page 45, line 16 - page 46, line 24	1-10

4

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 98/06725

Box I Observations where certain claims were f und unsearchable (C ntinuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 9 and 10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/06725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9702348 A	23-01-1997	AU 6238896 A	05-02-1997
		EP 0835314 A	15-04-1998
US 5182109 A	26-01-1993	JP 2243633 A	27-09-1990
		CA 1335571 A	16-05-1995
		DE 3911442 A	02-11-1989
		FR 2629717 A	13-10-1989
		GB 2217600 A,B	01-11-1989
		KR 9603378 B	09-03-1996
WO 9729771 A	21-08-1997	NONE	